Doctoral Thesis

MILK HYPERSENSITIVITY

Effects of Cow's Milk and its Processing on Gastrointestinal Symptoms and Delayed-Type Immune Responses

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Academic Dissertation

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ABBREVIATIONS

ANOVA	Analysis of variance
CCR	Chemokine receptor CC
CI_{95}	95% confidence intervals
CMA	Cow's milk allergy
CMSE	Cow's milk sensitive enteropathy
COLAP	Colonoscopic allergen provocation
GALT	Gut-associated lymphoid tissue
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
HLA	Human leukocyte antigen
IFN-γ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
LNH	Lymphonodular hyperplasia
sICAM-1	Soluble intercellular adhesion molecule 1
rt-PCR	Real-time polymerase chain reaction
TCRs	T-cell receptors
TGF-β	Transforming growth factor β
Th	T helper
TNF-α	Tumor necrosis factor α
tTG	Tissue transglutaminase

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals (**I-VI**). Some previously unpublished data are also presented.

I Paajanen L, Tuure T, Poussa T, Korpela R. No difference in symptoms during challenges with homogenized and unhomogenized cow's milk in subjects with subjective hypersensitivity to homogenized milk. *J Dairy Res* 2003;70:175-9.

II Korpela R, Paajanen L, Tuure T. Homogenization of milk has no effect on the gastrointestinal symptoms of lactose intolerant subjects. *Milk Sci Int (Milchwissenschaft)* 2005;60:3-6.

III Paajanen L, Tuure T, Vaarala O, Korpela R. Homogenization of milk has no effect on milk-specific antibodies in healthy adults. *Milk Sci Int (Milchwissenschaft)* 2005;60:239-41.

IV Paajanen L, Vaarala O, Karttunen R, Tuure T, Korpela R, Kokkonen J. Increased IFN-γ secretion from duodenal biopsy samples in delayed-type cow's milk allergy. *Pediatr Allergy Immunol* 2005;16:439-44.

V Paajanen L, Kokkonen J, Karttunen TJ, Tuure T, Korpela R, Vaarala O. Intestinal cytokine mRNA expression in delayed-type cow's milk allergy. *J Pediatr Gastroenterol Nutr* 2005, resubmitted.

VI Paajanen L, Korpela R, Tuure T, Honkanen J, Järvelä I, Ilonen J, Knip M, Vaarala O, Kokkonen J. Cow milk is not responsible for most gastrointestinal immune-like syndromes – evidence from a population-based study. *Am J Clin Nutr* 2005, in press.

ABSTRACT

The aim of this thesis was to study the effects of milk and its processing on gastrointestinal symptoms and immune responses, with special reference to conditions not related to immunoglobulin E.

Some people appear to experience cow's milk-related symptoms even though neither lactose intolerance nor cow's milk allergy (CMA) can be diagnosed. The cause of these symptoms is unclear, and apparently an unknown type of cow's milk hypersensitivity exists. It has been suggested that the processing of cow's milk may be involved in the induction of gastrointestinal symptoms. Homogenisation has been claimed as one possible cause. In this thesis, no difference in the tolerance to homogenised and unhomogenised milk was found, either in adults who had subjectively experienced better tolerance to unhomogenised than homogenised milk, or in lactose-intolerant adults, nor were any differences in the concentrations of milk protein-specific antibodies during open challenges with unhomogenised and homogenised milk found in milk-tolerant adults.

This thesis includes studies of immunological background and of the mechanism of delayed-type gastrointestinal CMA. The children with delayed CMA, diagnosed by an open cow's milk challenge and an endoscopic examination, showed local intestinal activation of both T helper 1 (Th1) and Th2 lymphocytes. The release of interferon γ and the expression of interleukin 6 (IL-6) and chemokine receptor CC 4 (CCR-4) mRNA were up-regulated in the intestinal mucosa of these children, and in those with delayed CMA who consumed milk, increased local secretion of IL-4 and IL-10 and decreased secretion of transforming growth factor β (TGF-β) were found.

The aim of the population-based study was to evaluate the occurrence of similar hypersensitivity against milk proteins in young adults with gastrointestinal complaints, as described in younger children earlier. However, no such cases with intestinal lymphonodular hyperplasia were found. Of the young adults, 10% reported major gastrointestinal complaints, 24% reported cow's milk-induced gastrointestinal symptoms and 13% did not drink any milk as such (n=827). However, in a blind challenge with a subgroup, cow's milk protein-induced symptoms were rare and similar to those of a placebo soy drink. The elevation of soluble intercellular adhesion molecule 1 (sICAM-1) in the plasma, and a tendency towards up-regulation of TGF-β and IL-12p35 mRNA expression in the intestinal mucosa of the symptomatic subjects who volunteered for clinical examination, indicate a possible immunological nature of the identified gastrointestinal disorder. The food-related gastrointestinal symptoms of young adults seemed to be caused by unspecific and unknown characteristics of altered mucosal immune response rather than being triggered by cow's milk, as is often suspected by the patients themselves. This new entity of intestinal immune-mediated disorder may be a self-perpetuating disease with fluctuating symptoms. An autoimmune nature of the

state, at least in a subgroup of the affected subjects, cannot be ruled out, and this hypothesis is supported by the observation that the human leukocyte antigen DQ*02 allele, which predisposes to autoimmunity, was almost twice as common among the symptomatic individuals as among the rest.

According to this series of studies, some young adults and some mature adults subjectively experience cow's milk-related symptoms, but often the symptoms cannot be objectively diagnosed, and homogenisation of milk does not seem to be the cause of them. In children, delayed-type CMA seems to be a local intestinal immune-activation state showing activation of both Th1 and Th2 lymphocytes. The findings of immunological activity in young adults imply the existence of a food-related gastrointestinal syndrome, which is not, however, induced by cow's milk.

TIIVISTELMÄ (Abstract in Finnish)

Tämän väitöskirjan tarkoituksena oli tutkia lehmänmaidon ja sen käsittelyn vaikutusta ruoansulatuskanavan oireisiin ja puolustusvasteisiin. Erityisen kiinnostuksen kohteena olivat reaktiot, jotka eivät liity immunoglobuliini E:hen.

Jotkut ihmiset kokevat saavansa lehmänmaidosta oireita, vaikka heillä ei voida osoittaa olevan laktoosi-intoleranssia eikä maitoallergiaa. Näiden oireiden syy on epäselvä, ja mitä ilmeisimmin tuntematon maitoyliherkkyyden muoto on olemassa. Maidon prosessoinnin on esitetty aiheuttavan vatsaoireita. Esimerkiksi maidon homogenointia on syytetty. Tässä väitöskirjassa homogenoidun ja homogenoimattoman maidon siedossa ei havaittu eroa aikuisilla, jotka kokivat sietävänsä homogenoimatonta paremmin kuin homogenoitua, eikä laktoosi-intoleranteilla aikuisilla. Maitoa sietävillä aikuisilla ei havaittu eroa maitoproteiinia kohtaan esiintyvien vasta-aineiden määrissä homogenoidun ja homogenoimattoman maidon nauttimisen aikana.

Tässä väitöskirjassa tutkittiin viivästyneen maitoallergian immunologista taustaa ja mekanismia. Lapsilla, joilla oli viivästynyt maitoallergia, havaittiin sekä auttaja T 1 (Th1) että Th2 lymfosyyttien paikallinen aktivoituminen suolen limakalvolla. Näillä lapsilla interferoni γ:n eritys, ja interleukiini (IL) 6:n ja kemokiinireseptori CC 4:n (CCR-4) mRNA:n ilmentyminen olivat lisääntyneet suolen limakalvolla. Lisäksi niillä maitoallergisilla lapsilla, jotka käyttivät maitoa, havaittiin IL-4:n ja IL-10:n paikallisen erityksen lisääntyneen ja transformoiva kasvutekijä β:n (TGF-β) erityksen vähentyneen.

Väestötutkimuksen tarkoituksena oli arvioida samanlaisen maitoproteiiniyliherkkyyden esiintymistä vatsaoireisilla nuorilla aikuisilla, mikä on aikaisemmin osoitettu nuoremmilla lapsilla. Tutkimuksessa ei kuitenkaan löytynyt yhtään vastaavaa tapausta, jossa olisi havaittu suolen imukudoslisää. Nuorista aikuisista 10 % kertoi kärsivänsä vakavista ruoansulatuskanavanoireista, 24 % raportoi saavansa oireita maidosta ja 13 % ei juonut maitoa (n=827). Osalle nuorista tehdyssä sokkokokeessa lehmänmaidon aiheuttamat oireet olivat harvinaisia ja lumesoijajuoman aiheuttamia oireita vastaavia. Intersellulaarisen adheesiomolekyyli 1:n (sICAM-1) lisääntyminen plasmassa ja suunta kohti TGF-β ja IL-12p35 mRNA:n ilmentymisen lisääntymistä ohutsuolen limakalvolla tukevat löydetyn ruoansulatuskanavan oireyhtymän immunologista luonnetta. Ruokaan liittyvät ruoansulatuskanavan oireet näyttävät aiheutuvan nuorilla aikuisilla epämääräisestä ja tuntemattomasta syystä eikä lehmänmaidosta, vaikka potilaat usein epäilevät lehmänmaidon yhteyttä oireisiin. Tämä uusi puolustusvasteen välittämä suoliston oireyhtymä voi olla itsestään syntyvä sairaus, jossa oireiden vaikeusaste vaihtelee. Taudin autoimmuuniluonnetta ei voida sulkea pois, ainakaan

osalla potilaista, ja teoriaa kannattaa havainto, että autoimmuunitauteihin liittyvä HL-antigeenin DQ*02 alleeli oli lähes kaksi kertaa yleisempi oireilevilla potilailla verrattuna muihin.

Tämän väitöskirjatutkimuksen mukaan osa nuorista aikuisista ja aikuisista kokee saavansa maidosta oireita, mutta oireita ei usein voida diagnosoida objektiivisesti, eikä maidon homogenoinnilla näytä olevan yhteyttä oireisiin. Lapsilla viivästynyt maitoallergia näyttää olevan puolustusvasteen tila, jossa sekä Th1 että Th2 lymfosyytit ovat aktivoituneet paikallisesti suolessa. Nuorilla aikuisilla havaittu puolustusvasteen aktivoituminen puoltaa ruokaan liittyvän ruoansulatuskanavan oireyhtymän esiintymistä, joka ei kuitenkaan näytä olevan lehmänmaidon aiheuttama.

INTRODUCTION

The prevalence of allergic diseases is increasing in western countries. The functions of the gut and the mucosal immune system are crucial in the induction of oral tolerance or allergic sensitisation to luminal antigens. Cow's milk allergy (CMA) is usually the first major food allergy, since cow's milk proteins are the first source of foreign antigens massively ingested in infancy. In several large clinical trials, the cumulative prevalence of allergy to cow's milk has been approximately 2-3% during the first years of life in the general population (Høst & Halken 1990, Schrander et al. 1993, Saarinen et al. 1999). The overall prognosis of CMA in infancy is good, with a remission rate of up to 85 or 90% (Høst 2002).

In recent years recovery from CMA has become a subject of controversy. Compared to the mainly immunoglobulin (Ig) E-mediated CMA of infants and small children, a new form of delayed-type gastrointestinal cow's milk hypersensitivity, also called cow's milk sensitive enteropathy, has been described in school-aged children and in adults, and it may be more common than previously thought (Bengtsson et al. 1996a, Pelto et al. 1998, Pelto et al. 1999, Ulanova et al. 2000, Kokkonen et al. 2001a, Lin et al. 2002, Magnusson et al. 2003, Kokkonen et al. 2004). After childhood, reactions towards milk are rarely IgE-mediated, and virtually only case reports of IgE-mediated CMA in adults exist.

Self-diagnosed cow's milk-related symptoms are commonly reported in questionnaires and interviews (Pelto et al. 1999, Haapalahti et al. 2004, Kokkonen et al. 2004). Some individuals claim that they are intolerant to cow's milk, even though neither lactose intolerance nor CMA can be diagnosed. Some declare that they tolerate raw untreated cow's milk and unhomogenised, pasteurised cow's milk but show reactions of intolerance to homogenised and pasteurised commercial cow's milk and dairy products. The parents of certain children who are allergic to cow's milk report the same phenomenon. However, in clinical studies, no difference in the tolerance of homogenised and unhomogenised cow's milk has been observed (Hansen et al. 1987, Høst et al. 1988, Pelto et al. 2000).

The aim of this study was to examine the effects of cow's milk and its processing on symptoms and intestinal immune activation in subjects with cow's milk intolerance or delayed-type CMA, and to study the occurrence of milk-related reactions and subjective symptoms in relation to verified milk hypersensitivity in young adults.

REVIEW OF THE LITERATURE

1 THE GUT IMMUNE SYSTEM

1.1 Mucosal immunology

The intestinal mucosa forms a major and critical barrier through which immunogenic particles and molecules such as food and microorganisms gain access to the immune system. The selective defence mechanisms of the gut make possible the absorption of essential substances, and simultaneously reduce the absorption and injurious effects of the immunogenic particles (Table 1). The intestinal absorption of food antigens is closely dependent on developmental and environmental factors, including the maturity of the intestinal mucosa, the sites of absorption (Peyer's patches), intestinal microbiota, and the presence of inflammation or infection (Heyman 2001).

Table 1 Barriers to macromolecular absorption. Antigen entry is prevented by immunological and non-specific mechanisms in the gastrointestinal tract as well as by the physiological structure of the epithelium itself (modified from Sanderson and Walker 1999).

Mechanism	Action
Immunological barrier	
Humoral: secretory IgA and IgM and other Ig	Neutralisation and removal of antigens
Cell-mediated: lymphocytes of epithelium and lamina propria	Specific local defence
Non-specific barrier	
Gastric acid	Dissolution of antigens
Digestive enzymes	Dissolution of antigens
Mucus coat and secretions	Inhibition of absorption
Humoral factors of innate immunity: lactoferrin, lysozyme, peroxidases	Dissolution of antigens
Normal microbiota	Dissolution of antigens, inhibition of absorption
Tight junctions of the epithelium	Inhibition of absorption
Hepatic filter	Removal of antigens

Ig, immunoglobulin

Classical effector cells of immune reactions, such as lymphocytes, dendritic cells, macrophages, eosinophils, mast cells and occasional neutrophils, are normally present or lie in close proximity to the epithelial layer. These cells and their intermediators constitute the immune system, which can be divided into innate immunity and adaptive immunity (Fig. 1).

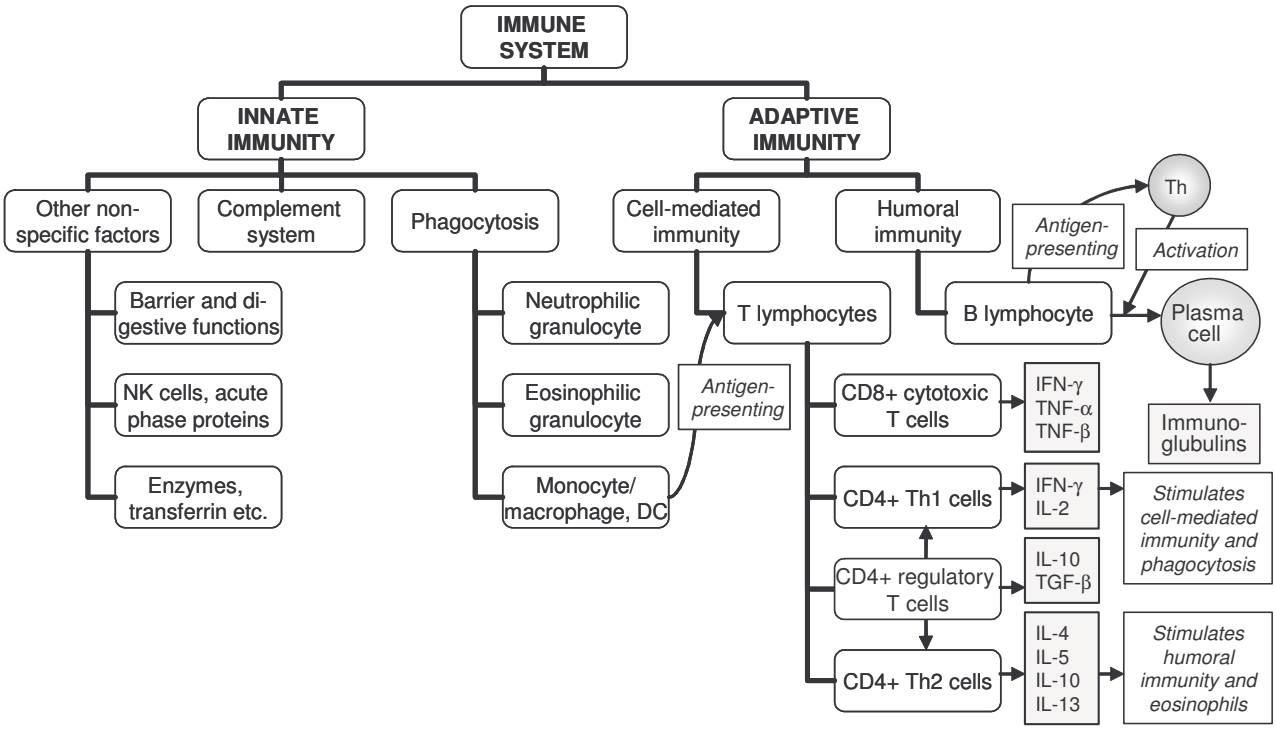

Figure 1 Diagrammatic representation of the human immune system. DC, dendritic cell; IFN-γ, interferon γ; IL, interleukin; NK cell, natural killer cell; Th, T helper lymphocyte; TNF, tumor necrosis factor.

Intestinal antigen handling determines the immune response to that antigen (Mayer 2000) (Fig. 2). Antigens are absorbed from the gut mainly through the epithelial cells, but some of large molecules may leak between the epithelial cells, and some antigens are absorbed intact through the M-cells and carried by antigen-presenting cells into the Peyer's patch follicles, an important factor in the development of tolerance (Heyman 2001). Across the epithelium, antigens are absorbed along two functional pathways. The main, degradative pathway reduces the immunogenicity of the antigen. A minor pathway allows the transport of intact proteins. Increased intestinal permeability and altered antigen transference across the intestinal mucosa has been reported in states with hyperreactivity to environmental antigens, such as atopic eczema and CMA (Jalonen 1991, Majamaa & Isolauri 1996).

Intact or partially digested antigens which pass through the epithelial barrier of the gut encounter the gut-associated lymphoid tissue (GALT) (Spahn & Kucharzik 2004). The GALT is a very well-developed immune network which protects the host from ingested pathogens and also prevents host adverse immune reactions to ingested dietary protein. The interaction of orally adminis-

tered food antigens with the GALT induces characteristic immunological responses such as the production of secretory IgA and the induction of oral tolerance.

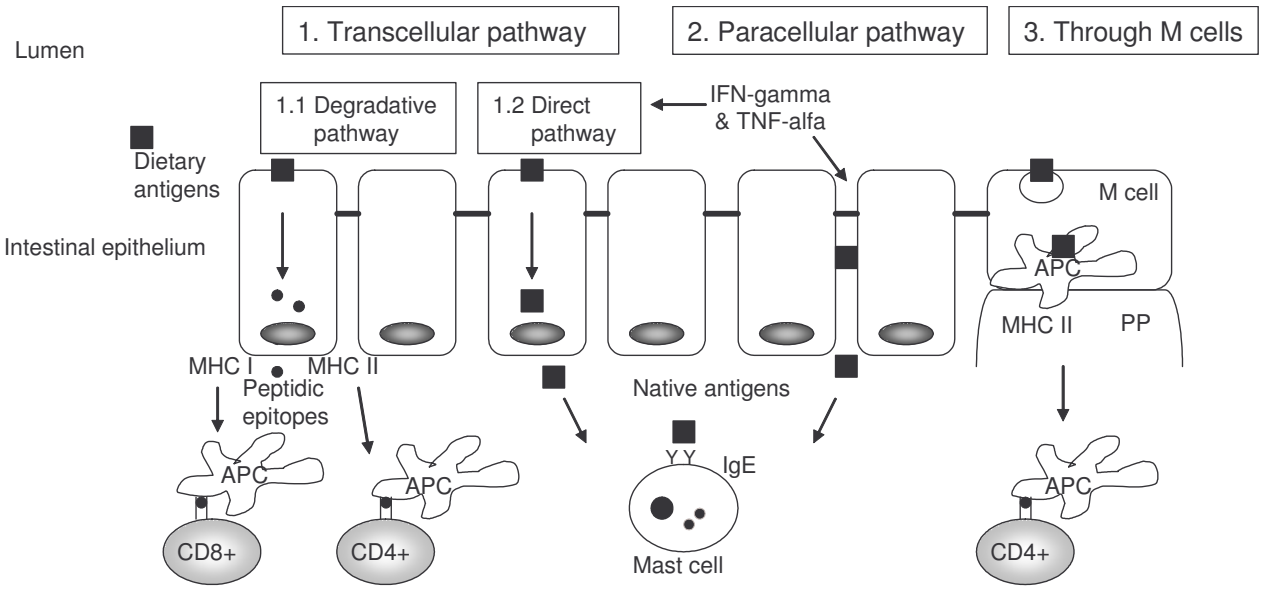

Figure 2 Diagrammatic representation of antigen absorption and outcome of antigen presentation, showing that the outcome differs depending on the inflammatory environment (modified from Heyman 2001, Strobel 2001). APC, antigen-presenting cell; IFN-γ, interferon γ; IgE, immunoglobulin E; MHC, major histocompatibility complex; PP, Peyer's patch; TNF-α, tumor necrosis factor α

The main inductive sites of the GALT are Peyer's patches, the organised lymphoid aggregates in the wall of the small and large intestine. The primary effector sites of mucosal immunity are the lamina propria, which contain T and B lymphocytes and other cells necessary for adaptive immune responses, and the epithelium, which contains a unique population of T cells called intraepithelial lymphocytes. T lymphocytes can be divided into those expressing αβ T-cell receptors (TCRs) for antigens and those expressing γδ TCRs. Lamina propria T cells mainly express αβ TCRs and CD4, while intraepithelial T cells contain a much higher percentage of $\gamma\delta^+$ T cells and have predominant CD8 expression, suggesting reaction to antigens in a class I major hiscompatibility complex restricted fashion (Lefrançois & Puddington 1999).

1.2 Local intestinal allergic reactions

Oral tolerance is defined as a state of immunological unresponsiveness to an antigen induced by the ingestion of that antigen (Strobel 2001). Oral tolerance is the immunological mechanism by which the mucosal immune system maintains unresponsiveness to the numerous antigens which might otherwise induce damaging immune responses. It appears to be mediated by several mechanisms, such as the antigen-specific generation of T cells which produce antigen non-specific regulatory cytokines. The development of oral tolerance is part of normal immunologic maturation, and IgE sensitisation to dietary antigens rather than tolerance may often occur in infancy, because of the immaturity of the gut or the intestinal lymphoid tissue or both. Intact antigens may penetrate the immature mucosa and induce an immunologic inflammatory reaction, which disappears when the infant grows up and the defence mechanisms develop. Increased intestinal permeability seems to be associated with the occurrence of mucosal inflammation and a lack of oral tolerance, i.e. allergic reactions (Jalonen 1991, Majamaa & Isolauri 1996, Kalach et al. 2001), celiac disease (Kuitunen & Savilahti 1996) and autoimmune diseases (Kuitunen et al. 2002), and is also found in premature infants (Boehm et al. 1992). In healthy full-term infants, growth factors in colostrum milk activate the maturation of the intestinal mucosa so that gut closure and a normal permeability are rapidly observed after birth (Vukavic 1984, Catassi et al. 1995). During the first months of life the production of secretory IgA is insufficient in the gut of an infant, and instead, the secretory IgA of human milk neutralises antigens.

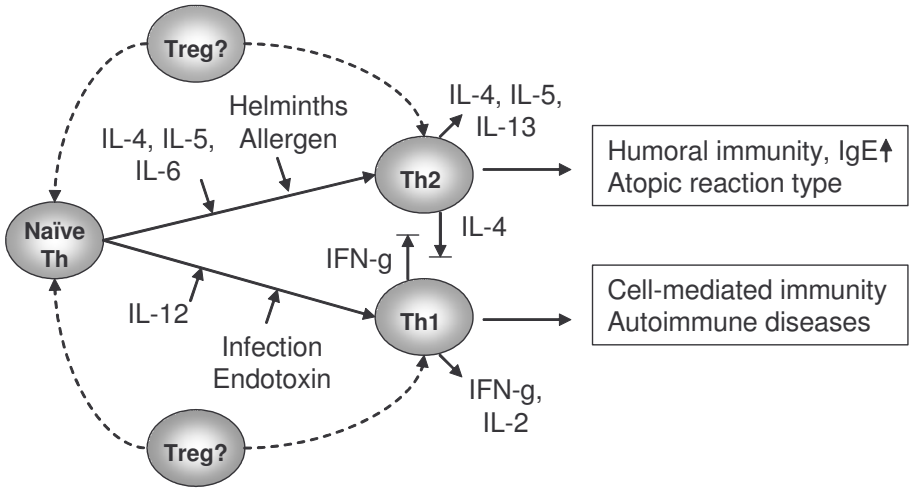

Figure 3 Differentation of naïve $CD4^+$ T cells in response to environmental factors, cytokines and the possible controlling effect of regulative T cells (modified from Ngoc et al. 2005). Th, T helper lymphocyte; Treg, T regulative lymphocyte

Interactions between T and B lymphocytes and certain regulatory cytokines influence the initiation and maintenance of allergic responses (Fig. 3). The immunological regulatory system of an infant favours allergic reaction type, because the T helper lymphocyte 1 (Th1) reactions are restrained during the embryonic stage (Dealtry et al. 2000). Upon antigen contact, $CD4^+$ Th2 cells reinforce humoral immunity through the activation of B cells and the production of interleukin 4 (IL-4), IL-5 and IL-10, which direct the immunoglobulin class switch to IgE and IgG1 and stimulate eosinophils (Kuhn et al. 1991, Torres et al. 2004). IL-13, which has the same homology as IL-4, also stimulates IgE production and immediate-type hypersensitivity reactions (Hajoui et al. 2004). In atopic infants the maturation of oral tolerance is prevented and new antigen contacts reinforce the Th2-type response. Healthy infants may also produce specific IgE antibodies against dietary antigens during the first months of life. However, soon antigen, microbe and virus stimulations induce Th1-type cytokines, and Th1/Th2 balance is achieved through immune deviation. Th1 cells mainly produce interferon γ (IFN-γ) and IL-2 upon activation and reinforce cell-mediated immunity, phagocytosis and delayed-type hypersensitivity tissue damage. IFN-γ balances the effects of IL-4 and antagonises the production of IgE (So et al. 2000).

The immune mechanisms involved in allergy are complex and cannot be explained by a simple shift from Th2 to Th1 immune responses. If reduced microbial exposure impaired the immune deviation from Th2 to Th1, one would not expect to see an increased prevalence of both autoimmune diseases (Th1-dominant immune responses) and allergic diseases (Th2-dominant immune responses). In fact, allergic sensitisation may be due to inadequate regulatory responses of the T cells rather than Th1/Th2 imbalance (Yazdanbakhsh et al. 2002, Karlsson et al. 2005), and the dominant immunological abnormality in the small bowel of food-allergic children may be a failure to establish normal numbers of the transforming growth factor β (TGF-β) which produces regulatory cells (Chung et al. 2002, Pérez-Machado et al. 2003). The reduction of intestinal regulatory lymphocyte numbers may lead to a lack of bystander tolerance, and thus a tendency for multiple sensitisations (Groux & Powrie 1999, Strobel 2001). A recent study showed that induction of oral tolerance in children with CMA was associated with the appearance of circulating $CD4^+CD25^+$ regulatory T cells capable of suppressing the effector T cells generated by oral administration of dietary antigens (Karlsson et al. 2005).

The possible role of the intestinal microbiota in the maturation of the immune system in infants and in the development of oral tolerance against foods has received considerable attention. According to animal and *in vitro* studies, the intestinal microbiota seems to stimulate the maturation of immune responses (Smits et al. 2004), and probiotic strains of lactobacilli are of particular in-

terest in this respect (Vaarala 2003). Differences in intestinal *Bifidobacterium* flora composition have been reported between infants in Sweden, with a high incidence of atopic disease, and in Estonia, with low incidence, and between allergic and healthy children (Sepp et al. 1997, Björkstén et al. 1999, Björkstén et al. 2001, Ouwehand et al. 2001, Watanabe et al. 2003). The reduction of bifidobacteria has been shown to precede the development of atopic disease, suggesting an essential role of the balance of indigenous intestinal bacteria for the maturation of human immunity to a non-atopic mode (Kalliomäki et al. 2001a). Indeed, a probiotic *Lactobacillus* strain GG has been shown to reduce symptoms in infants with atopic dermatitis (Majamaa & Isolauri 1997, Isolauri et al. 2000, Viljanen et al. 2005a), and to prevent early atopic disease in children at high risk (Kalliomäki et al. 2001b, Rautava et al. 2002). The anti-allergenic effects of probiotics may be mediated by the stimulation of Th1 cytokines (Maassen et al. 2000, Pohjavuori et al. 2004, Adel-Patient et al. 2005), or of secretory gut IgA (Ibnou-Zekri et al. 2003, Viljanen et al. 2005b), and also by the induction of sufficient responses, even low-grade inflammation, in the gut epithelium and macrophages in order to allow the effective generation of regulatory lymphocyte populations (Isolauri et al. 2000, Paganelli et al. 2002, Viljanen et al. 2005c).

These findings of microbiota, probiotics and atopic diseases support a so-called hygiene hypothesis: the rapid increase in atopy may be related to less exposure to environmental microbes and infections in infancy, because the immune response to microbial antigens drives the expression of Th1 cytokines and counterbalances Th2 cytokine production, continuation of which might lead to enhanced IgE production and atopic diseases (von Mutius 1998, Aalberse & Platts-Mills 2004, Rautava et al. 2004, Romagnani 2004, Williams et al. 2004). However, studies opposed to the hygiene hypothesis also exist: in developing countries, microbes and infections do not seem to protect from atopy (Chai et al. 2004, Kramer et al. 2004).

1.3 Immune mechanisms of gastrointestinal symptoms

Many food-provoked symptoms such as nausea, vomiting, abdominal cramps, distension and diarrhoea are presumed to originate from the gastrointestinal tract. It is obviously difficult to find a specific immunological response and to diagnose a specific disease which might cause such common and generalised symptoms. The intensity of the symptoms is difficult to quantify objectively. The quantity of intestinal gas (Chami et al. 1991, Koide et al. 2000) and the dilatation of the bowel (Whitehead et al. 1990) have been measured, often with poor correlation to the symptoms, and therefore a written symptom record is still the most common way of assessing the symptoms. Furthermore, some patients may have abnormal pain response to gut distension, disordered intestinal

motility, an altered contractile activity of the gut, and an altered compliance of the gut related to wall tension or muscle tone, or they may perceive intestinal stimuli diffusedly (Whitehead et al. 1990, Accarino et al. 1995).

The monocytes/macrophages and other antigen-presenting cells are important in processing antigens and presenting them to the lymphocytes in such a way that an appropriate immune response is triggered. Intestinal diseases are often associated with an increased permeability to macromolecular food antigens which, after penetration to the intestinal lumen, can stimulate the underlying immune system. The release of cytokines and inflammatory mediators further enhances leakage through the epithelial barrier, leading to a vicious circle of inflammation (Chung et al. 2002). The well-known pro-inflammatory cytokine IFN-γ has been shown to disrupt tight junctions and to increase the paracellular permeability of the intestinal epithelium (Adams et al. 1993, Ferrier et al. 2003).

Celiac disease occurs in genetically susceptible individuals expressing the human leukocyte antigen (HLA) alleles HLA-DQ2 (DQA1*05-DQB1*02) or HLA-DQ8 (DQA1*0301-DQB1*0302) haplotype (Green & Jabri 2003, Koning 2003). In celiac disease, it is mainly Th1-type inflammatory IFN-γ but also Th2-type cytokines, e.g. IL-4, and macrophage-derived cytokines, e.g. tumor necrosis factor α (TNF-α), that have been shown to be up-regulated, and there is a massive increase of intraepithelial $CD3^+$, $\alpha\beta^+$ and $\gamma\delta^+$ T cells (Kontakou et al. 1995, Forsberg et al. 2002, Olaussen et al. 2002, Westerholm-Ormio et al. 2002, Veres et al. 2003). The activation of the immune cascades is much weaker and less well-known in delayed-type gastrointestinal food allergy; however, an accumulation of lymphoid cells in the form of nodules and a mild increase of $\gamma\delta^+$ T cells has been reported (Spencer et al. 1991, Kokkonen et al. 2000, Kokkonen et al. 2001b). Lacking villous atrophy and/or the accumulation of mononuclear cells in the lamina propria, the symptoms of delayed-type food allergy have been thought to originate from a cytokine imbalance, possibly an up-regulation of both Th2- and Th1-type cytokines (Wakefield et al. 2000, Veres et al. 2003). The duodenal biopsies of children with gastrointestinal food allergy have shown up-regulation of IFN-γ and, to a lesser extent, IL-4 secreting cells (Hauer et al. 1997, Veres et al. 2003). Even though food-hypersensitive adults rarely have systemic food-specific IgE, they may have local allergic reaction in the intestinal mucosa, seen as high numbers of IgE-bearing cells, activated eosinophils and T cells (Lin et al. 2002).

2 CLASSIFICATION OF ADVERSE REACTIONS TO FOOD

The mechanisms of adverse reactions to food are multiple (Fig. 4). **Food aversion** is a psychological problem connected to the ingestion of particular foods, and would not occur if the food was presented in disguised form. **Food intolerance** is an unpleasant reproducible reaction to a specific food, which lacks either a psychological or a known immunological basis, and is based on other defined mechanisms or factors, such as pharmacological ones (caffeine), enzyme deficiency (lactase, sucrase), or non-specific histamine release (strawberries, see chapter 2.2). **Food hypersensitivity/allergy** is defined as an adverse reaction to food mediated by dietary antigens, where an involvement of the immune system can be demonstrated (see chapter 2.1).

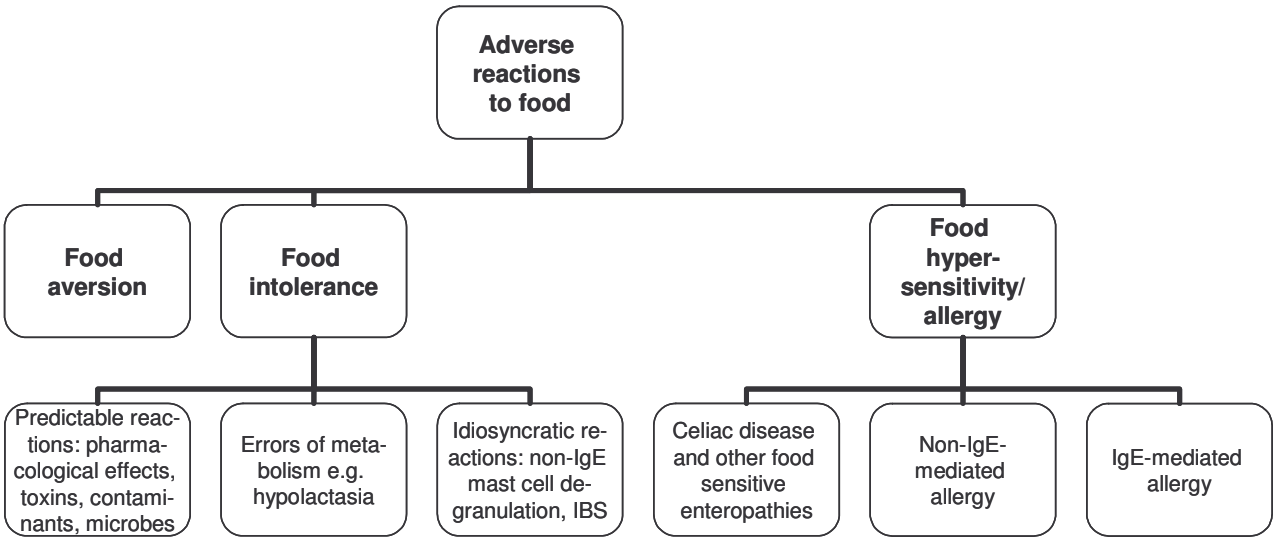

Figure 4 Classification of adverse reactions to food (modified from Fickling & Robertson 2002a, Fickling & Robertson 2002b). IBS, irritable bowel syndrome

Despite such apparent clear-cut definitions, diagnosis at clinical level is far more difficult to establish. For example, an obvious gap exists between self-reported food-related allergic symptoms and those that can be objectively confirmed by a double-blind placebo-controlled food challenge (Jansen et al. 1994, Young et al. 1994, Roehr et al. 2004, Zuberbier et al. 2004). Nor is it always possible to demonstrate the involvement of the immune system in non-IgE-mediated allergy.

2.1 Food allergy

Food allergy is triggered by an aberrant immune response elicited by the oral administration of dietary antigens. Systemic exposure to an antigenic stimulus leads to the development of specific antibodies and of cell-mediated immunity. In most cases, continuous exposure leads to tolerance, the specific state of unresponsiveness. Allergic reactions are traditionally classified under four types of hypersensitivity reaction which may lead to tissue damage, as described by Coombs and Gell (see Britton 2002, Hay & Westwood 2002, Male 2002, Platts-Mills 2002). It is not clear whether all four types of reaction are involved in the pathogenesis of food allergy, either in the gut itself or in remote organs. More than one mechanism may be involved in any allergic reaction, but the most plausible mechanisms are IgE-mediated reactions (Type I), and the non-IgE-mediated activation of T lymphocytes (Type IV): allergies may be exclusively IgE-mediated, partially IgE-mediated or exclusively cell-mediated (Sampson 2001).

Type I, immediate anaphylactic hypersensitivity is characterised by the production of IgE antibodies against foreign proteins (Platts-Mills 2002). IgE antibodies bind to high-affinity FcεRI receptors on mast cells and basophils. When an allergen binds between two IgE antibodies, it induces degranulation of a mast cell/basophil, which leads to the rapid release of histamine and the more gradual release of other mediators such as leukotrienes and cytokines. The combined effect of these agents is to constrict smooth muscle, dilate capillaries and induce cell infiltration. This mechanism underlies the common problem of atopic allergy.

Type II, antibody-dependent cytotoxic hypersensitivity is an important part of the body's normal humoral immune response (Male 2002). IgG or IgM antibodies identify cell-surface antigens on foreign antigens or an individual's own cells, such as transformed red blood cells, then activate the complement system and damage the cell. Killer cells, platelets, neutrophils, eosinophils and mononuclear phagocyte cells have receptors for IgG and the activated C3b components of the complement system, and can therefore cause Type II lytic damage to the target cells.

Type III, immune-complex-mediated hypersensitivity is complement and effector-cell mediated tissue damage (Hay & Westwood 2002). Food antigens are often absorbed from the gut in small amounts and may form immune complexes with specific antibodies in the circulation, especially in atopic subjects (Paganelli et al. 1981). Generally, these complexes are effectively removed by the mononuclear phagocyte system, but occasionally they persist, establish themselves in tissues and organs, and cause acute inflammation and tissue damage by activating the complement system.

Type IV, delayed cell-mediated hypersensitivity reactions take more than 12 hours to develop (Britton 2002). T cells identify antigens, and the antigen-sensitised T cells produce cytokines and other soluble factors which mediate the hypersensitivity reaction, or else they develop cytotoxicity. Th cell-activated macrophages destroy intracellular bacteria by releasing inflammatory mediators. Activated cytotoxic T cells and natural killer cells destroy virus-infected cells and transformed human cells, i.e. cancer cells and tissue transplants. Tissue damage occurs as a result of persistent antigenic stimulation, either because of continuing infection or because of autoimmunisation. Type IV hypersensitivity has been classified under three varieties: contact hypersensitivity and tuberculin-type hypersensitivity, which both occur within 3 days of a challenge; and granulomatous hypersensitivity reactions, which develop over a period of 21-28 days and are clinically the most serious of the Type IV responses. More than one type of delayed hypersensitivity may follow a single antigenic challenge, and reactions may overlap.

2.2 Food intolerance

Adverse reactions to food which do not involve the immune system are described as food intolerances. Fickling and Robertson (2002b) have classified non-immunological adverse reactions into three main groups: 1) predictable reaction, 2) errors of metabolism, and 3) idiosyncratic reactions.

Predictable adverse reactions to food are expected to occur in any individual exposed to that food, although individual variations in susceptibility may exist. Examples include the toxic effect of non-nutrients contained in foods (e.g. mushroom toxins, bean lectins, lead, cadmium); microbial contamination causing gastroenteritis; and the pharmacological effect of foods containing caffeine, salt, alcohol, natural laxatives, or biogenic amines (e.g. histamine, tyramine) (Denaro et al. 1991, Morrow et al. 1991, Kanny et al. 1993, Kanny et al. 1996).

The most common **error of metabolism** is hypolactasia, in which the genetically determined reduction of lactase activity occurs after weaning (see chapter 3.4). Primary enzyme deficiencies at the time of birth are very rare, and comprise neonate hypolactasia and deficiencies of sucrase-isomaltase, trehalase, or enteropeptidase as well as other rare deficiencies (Arola et al. 1999, Belmont et al. 2002, Holzinger et al. 2002, Ritz et al. 2003).

In **idiosyncratic reactions,** the mechanism of intolerance may be unclear but does not involve the immune system. Certain food polypeptides may bind to mast cell IgE receptors and induce the non-immunological release of histamine and other chemical mediators from mast cells. Strawberries are the most common non-specific histamine releasers, but egg white, crustaceans, fish, tomatoes and the proteases of pineapple and papaya have also been reported (Kanny & Moneret-

Vautrin 2002). These 'pseudoallergens' may exacerbate atopic dermatitis or chronic urticaria (Worm et al. 2000, Buhner et al. 2004). Subjects with irritable bowel syndrome often relate the exacerbation of their symptoms to certain foods. The mechanisms and evidence of these reactions are unclear, and dietary manipulation frequently yields rather disappointing results (Dapoigny et al. 2003, Soares et al. 2004, Whorwell & Lea 2004). However, some studies suggest that food intolerance is the cause of irritable bowel syndrome in approximately 50% of cases (Nanda et al. 1989). Indigestible carbohydrates, such as sweeteners (Born et al. 1994, Storey et al. 2002), fructo-oligosaccharides (Briet et al. 1995, Teuri et al. 1999) and galacto-oligosaccharides (Teuri et al. 1998), induce gastrointestinal symptoms in certain subjects, possibly depending on the capability of the subject's intestinal microbiota to produce gases, and the sensitivity of the subject to the feeling of gastrointestinal swelling.

3 ADVERSE REACTIONS TO COW'S MILK

3.1 Different types of cow's milk allergy

CMA before school age

CMA is usually the first major food allergy, since cow's milk proteins are the first source of foreign antigens massively ingested in infancy. In several large clinical trials, the cumulative prevalence of allergy to cow's milk has been approximately 2-3% during the first years of life in the general population (Jakobsson & Lindberg 1979, Hide & Guyer 1983, Bock 1987, Høst & Halken 1990, Schrander et al. 1993, Saarinen et al. 1999). In atopic infants, however, the prevalence is up to 50% (Sampson & McCaskill 1985, Isolauri & Turjanmaa 1996, Niggemann et al. 1999). CMA has been reported even in exclusively breast-fed infants (Høst et al. 1988, Isolauri et al. 1999, Järvinen et al. 1999, Österlund et al. 2004a). The majority of paediatric patients have symptoms from two or more organ systems: approximately 50-60% have cutaneous, 50-60% gastrointestinal and 20-30% respiratory symptoms (Høst 2002). In exclusively breast-fed infants with CMA, severe atopic eczema is a predominant symptom. In infants under the age of one year, CMA is reportedly IgE-mediated in 57-64% of the cases (Vanto et al. 1999, Saarinen & Savilahti 2000). The overall prognosis of CMA in infancy is good, with a remission rate of 85 or 90% by 3 years of age (Høst & Halken 1990, Høst 2002), non-IgE-mediated reactions being the quickest to recover (Vanto et al. 2004).

The basic treatment of CMA is complete avoidance of cow's milk protein. The main allergens in cow's milk protein are casein and whey proteins, β-lactoglobulin and α-lactalbumin (Jensen 1995). The protein fraction of cow's milk consists of at least 20-30 different proteins, all possible

allergens, and therefore hydrolysation of some main allergens does not help the majority of allergic patients. Even though proof from repeated trials of double-blind placebo-controlled cow's milk elimination diets is lacking, an elimination diet is believed to alleviate symptoms, preserve intestinal integrity, prevent aberrant antigen absorption and reverse the disturbance of humoral and cell-mediated immune response (Isolauri et al. 1992, Majamaa et al. 1996). However, the positive effect of an elimination diet may partially be explained by the fact that many children grow out of their allergies, and a cow's milk elimination diet has been shown to be equally efficacious in the treatment of atopic dermatitis, both in children tolerant to cow's milk and in children with CMA (Viljanen et al. 2005ac). According to the latest view, a continuous allergen load might maintain tolerance: in one case study, long term milk protein elimination led to fatal milk protein hypersensitivity (Barbi et al. 2004); and a successful oral desensitisation to milk protein has been reported in children with IgE-mediated CMA (Meglio et al. 2004). The concept of the maintaining of oral tolerance vs. an elimination diet needs further research. Unnecessary food avoidance should be discouraged.

CMA in school-aged children and adults

In most textbooks CMA is considered to be rare in adults. Only a restricted number of studies on the immunological mechanisms of adult CMA exist, supported by either double-blind, placebo-controlled milk challenges or the open challenge procedure (Nørgaard & Bindslev-Jensen 1992, Nørgaard et al. 1995, Bengtsson et al. 1996b, Bengtsson et al. 1996a, Bengtsson et al. 1997, Werfel et al. 1997, Little et al. 1998, Ulanova et al. 2000, Lin et al. 2002, Magnusson et al. 2003). In recent years recovery from CMA has become a subject of controversy. Compared to the mainly IgE-mediated CMA of infants and small children, a new form of delayed-type gastrointestinal cow's milk hypersensitivity, also known as cow's milk sensitive enteropathy, has been described in school-aged children and adults, and it may be more common than previously thought (Bengtsson et al. 1996b, Bengtsson et al. 1996a, Bengtsson et al. 1997, Pelto et al. 1998, Pelto et al. 1999, Ulanova et al. 2000, Kokkonen et al. 2001a, Lin et al. 2002, Magnusson et al. 2003, Kokkonen et al. 2004). After childhood, reactions to milk are rarely IgE-mediated, and virtually only case reports of IgE-mediated CMA in adults exist. In one study, only 10% of the children with CMA in childhood had IgE-mediated reactions to milk protein at school age; however, half the children still reported gastrointestinal symptoms related to the ingestion of cow's milk protein (Kokkonen et al. 2001a).

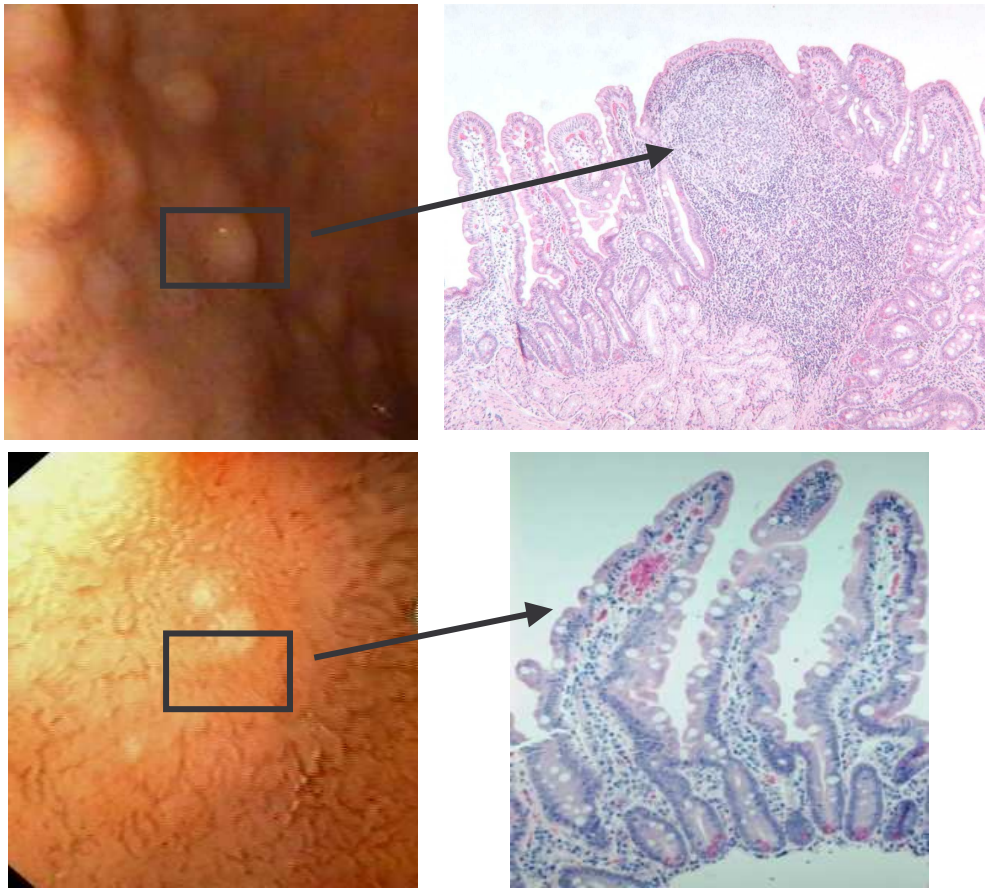

Figure 5 Top: In a 10-year-old boy who reacted in an elimination-challenge test to cow's milk, an endoscopic view on the mucosa of the duodenal bulb shows numerous lymphoid nodules (left). A histology of the biopsy reveals a lymphoid nodule with a germinal centre but otherwise normal mucosal architecture with tall and slender villi (right). Below: Normal endoscopic view, nice villous architecture and slender, tall villi without lymphoid aggregations in a healthy individual. Photographs provided by Dr. Jorma Kokkonen (Oulu, Finland).

The up-regulation of the local immune T cell responses *in situ* on the mucosa may cause milk-related gastrointestinal reactions in delayed-type gastrointestinal CMA. In school-aged children, delayed CMA seems to be characterised by an endoscopic finding of patchy, sometimes diffuse, lymphonodular hyperplasia (LNH) involving the upper duodenum (Fig. 5), the terminal ileum and/or the colon, and by slightly elevated densities of intraepithelial $\gamma\delta^+$ T cells (Kokkonen 1999, Kokkonen et al. 2000, Kokkonen et al. 2001c, Kokkonen et al. 2001b, Turunen et al. 2004). Typical features in a microscopic examination of the biopsy samples are increased frequency of lymphoid follicles with germinal centres and sometimes mildly increased density of eosinophilic leukocytes, but neither villous atrophy nor the increase of mononuclear cell infiltration in lamina propria have been reported. These features are markedly different from those in subjects with celiac

disease or in small children with food allergy. Indeed, in one study children who reacted in a blind challenge exhibited LNH of the duodenal bulb but failed to show signs of markers for atopic food allergy as measured by positive skin-prick tests or food-specific IgE-class antibodies (Kokkonen et al. 2001b). In addition to the fact that non-invasive diagnosis is difficult, endoscopic assessment is hampered since the mucosal lesions associated with the immune responses may vary in severity and extent, and they may be patchy, involving various segments of the gastrointestinal tract.

3.2 Diagnosis of cow's milk allergy

As no single laboratory test is diagnostic for either immediate or delayed-type CMA, the diagnosis still has to be based on strict, well-defined milk elimination and challenge procedures, and immunologic measurements may aid the diagnosis (Table 2). The diagnosis of gastrointestinal cow's milk hypersensitivity is by no means simple. The main symptoms are common to many conditions and it is difficult to distinguish between unspecific milk intolerance and milk allergy. Clinical history is crucial in the diagnosis, especially in infants who may have ingested only a few solid foods.

Table 2 Diagnosis of immunoglobulin E (IgE)-mediated and delayed-type cow's milk allergy, and experimental methods related to mechanism studies (modified from Terho et al. 1999).

Diagnostic and experimental methods
Clinical history
Elimination diet
Challenge, open/blind
Skin prick test
Measurement of milk protein-specific IgE from serum
Atopy patch test
Experimental methods
Measurement of local/systemic cytokines
Measurement of gut inflammation and permeability
Local challenge during endoscopy
Histamine release test

In young infants, open controlled challenges have been shown to be reliable when performed under professional observation in a hospital (Høst & Halken 1990, Niggemann et al. 1994, Isolauri & Turjanmaa 1996). In children over 1-2 years of age and in adults, the double-blind placebo-controlled food challenge is considered the gold standard for exclusion of psychological or causal

reactions (Høst & Halken 1990, Niggemann et al. 1994), but is often too laborious in clinical work (Kaila et al. 2000). In patients with delayed reactions, a placebo-controlled food challenge would be the best method of diagnosis (Bindslev-Jensen et al. 2004), but this is often not practical in clinical work. In adults in particular it may be difficult to distinguish gastrointestinal allergies from other gastrointestinal symptoms such as those of lactose intolerance or irritable bowel syndrome.

In early infancy and especially in breast-fed infants who develop immediate reactions to cow's milk, the presence of a specific elevation of IgE antibodies to cow's milk or a skin prick test for cow's milk may have diagnostic value. According to reports, the specificity of the skin prick test varies greatly, from 50 to 99%, and sensitivity, from 14 to 78%, when the cut-off size of the wheal diameter is set at 3 mm (Majamaa et al. 1999a, Vanto et al. 1999, Roehr et al. 2001, Saarinen et al. 2001, Strömberg 2002, Rancé 2004). In these particular studies, the atopy patch test was found to have better specificity (71 to 100%), especially in patients with skin symptoms, but its sensitivity for identifying all the disease cases was comparatively low (from 26 to 89%). When quantitatively assessed by the CAP System FEIA, cow's milk specific IgE antibody titres of over 32 kU/l have been reported to predict IgE-mediated CMA with as high as 95% certainty in atopic patients (Sampson & Ho 1997). However, this finding was not supported by a recent study in which much higher cow's milk-specific IgE titres (88.8 kU/l) were needed for predicting CMA with 90% probability, and the authors concluded that no meaningful predictive decision point could be calculated for predicting CMA (Celik-Bilgili et al. 2005). Skin tests are rarely useful in adults (Nørgaard & Bindslev-Jensen 1992). Increased IgA and IgG milk antibodies are not diagnostic, but merely a sign that milk has been ingested. Basophil histamine release is more frequently measured in other food allergies (Hansen et al. 2003, Østerballe et al. 2003) than in CMA (Prahl et al. 1988), and has not usually been found to be more predictive than skin prick testing or milk-specific IgE testing.

Experimental diagnostic methods in CMA

Besides the diagnostic methods summarised above, a number of experimental methods have attracted scientific interest and may be helpful for studies of the mechanisms of CMA. Exposure to cow's milk enhances mucosal permeability and elicits inflammation in the gut in infants with CMA (Majamaa & Isolauri 1996), possibly through the synergistic action of TNF-α and IFN-γ (Heyman et al. 1994, Benlounes et al. 1996). For example, a lactulose and mannitol permeability test can be used in clinical work (Jalonen 1991, Kalach et al. 2001). Permeability can also be measured from intestinal biopsy samples *in vitro* in the so-called Ussing chamber, which may also include an antigen challenge (Heyman et al. 1994, Majamaa & Isolauri 1996, Terpend et al. 1999).

Markers of inflammation in the faeces, such as eosinophil protein X, eosinophil cationic protein, IgA, TNF-α and α-antitrypsin, have been reported as non-invasive indicators of intestinal inflammation in atopic infants with CMA (Majamaa et al. 1996, Majamaa et al. 1999b, Majamaa et al. 2001, Saarinen et al. 2002). Increased eosinophil activation, such as increased eosinophil protein X in faecal samples (Magnusson et al. 2003) and activated eosinophils in small intestinal biopsy specimens (Lin et al. 2002, Schwab et al. 2003), has also been reported in food allergic adults. Eosinophil activation may be used for detecting ongoing clinical or subclinical chronic intestinal inflammation.

Cell-mediated reactions may be studied with the lymphocyte profileration test, which detects the proliferation activity of the patient's lymphocytes when they are stimulated with milk antigens *in vitro* (Beyer et al. 2002). Cytokines released by lymphocytes *in vitro* to cell-culture media can be measured with an enzyme-linked immunosorbent assay (ELISA) (Heyman et al. 1994, Benlounes et al. 1996, Beyer et al. 2002). An enzyme-linked immunosorbent spot (ELISPOT) assay can be used to study the frequency of cells secreting certain cytokines (Suomalainen & Isolauri 1994, Hauer et al. 1997, Järvinen et al. 1999).

Chronic gastrointestinal allergy may cause marked mucosal injury, histological changes and the up-regulation of intraepithelial T cells in the small intestine and the colon (Kokkonen 1999, Kokkonen et al. 1999, Kokkonen et al. 2000, Kokkonen et al. 2001c, Kokkonen et al. 2001b, Veres et al. 2003). Mucosal changes during a local antigen challenge can be measured by endoscopy; challenges both of stomach and of colon ('COLAP', colonoscopic allergen provocation) have been reported (Bischoff et al. 1997a, Bischoff et al. 1997b). However, the procedure is laborious and the results seem to be no more reliable than those achieved with a placebo-controlled food challenge. The measurement of serum complement changes during a placebo-controlled food challenge (Martin et al. 1984, Isolauri et al. 1997, Pelto et al. 2000), cell-surface markers of antigen specific T cells (Schade et al. 2002), and T-cell signal transduction (Österlund et al. 2003) have all attracted scientific interest.

3.3 Lactose intolerance

In lactase deficiency the activity or concentration of the lactose cleaving enzyme β-galactosidase, also called lactase, in the brush border of the small intestinal mucosa is insufficient. This hypolactasia causes insufficient digestion of lactose, the major carbohydrate of milk, a phenomenon called lactose malabsorption or lactose maldigestion. As reviewed by Sahi (1994), lactose maldigestion affects approximately 60% of the world's adult population, the prevalence varying in Europe from

2% in Scandinavia to 70% in Italy, 70-90% in South America, Africa and Asia, and reaching 100% in some Asian countries.

The forms of lactose maldigestion are 1) lactase non-persistence, 2) secondary lactose maldigestion, and 3) rare congenital lactase deficiency. In **lactase non-persistence**, also called adult-type lactose maldigestion, lactase activity is high at birth, decreases in a genetically programmed way in childhood and adolescence, and remains low in adulthood, which is the normal physiological situation for humans and other mammals. In populations where lactase non-persistence is predominant, the loss of lactase begins soon after weaning, and vice versa – in populations with low prevalence of lactose maldigestion, it develops later in adolescence or even in early adulthood (Sahi et al. 1983, Sahi 1994). Lactase is found at the tip of the intestinal villi, and is therefore vulnerable to intestinal diseases, inflammation and chemotherapy, leading to a **secondary form of lactose maldigestion**. Typically, lactase activity returns after recovery from the original disease (e.g. celiac disease, Crohn's disease, enteritis) and after the discontinuation of chemotherapy (Bode & Gudmand-Hoyer 1988, Murphy et al. 2002, Österlund et al. 2004b). A small intestinal resection may cause irreversible secondary lactose maldigestion. **Congenital lactase deficiency** is an extremely rare inheritable genetic defect, which is apparent immediately after birth (Savilahti et al. 1983).

Hypolactasia accompanied by clinical symptoms such as bloating, flatulence, nausea, diarrhoea, and abdominal pain is called lactose intolerance. The symptoms occur when undigested lactose passes to the large intestine, where it serves as a fermentable substrate for the microbiota and osmotically increases the flow of water into the lumen. The intensity of the symptoms depends on the amount of lactose ingested, on individual sensitivity, the rate of gastric emptying, gastrointestinal transit time, and the pattern of microbiota in the large intestine. Ingestion of 50 g lactose, the amount commonly used in clinical tolerance tests, causes symptoms in 80-100% of lactose maldigesters, whereas the ingestion of a glass of milk (200-250 ml) causes symptoms to only 30-50% (Vesa et al. 2000). For some unknown reason, a small percentage of maldigesters remain symptom-free even after the ingestion of large amounts of lactose. Symptoms of lactose intolerance can be reduced by food and meal pattern choices and by the consumption of low-lactose and lactose-free dairy products. Total avoidance of dairy products often results in poor calcium intake and an increased risk of fractures; so lactose intolerance is associated with reduced bone mineral density and may predispose to bone fractures (Jackson & Savaiano 2001, Kudlacek et al. 2002, Obermayer-Pietsch et al. 2004). Self-described "lactose-intolerant" individuals may restrict their dairy and calcium intake without real clinical need, and are at risk of osteoporosis and bone fractures (Savaiano 2003).

Lactose digestion can be measured by direct or indirect methods (Arola 1994). The direct methods – the measurement of mucosal disaccharidases, and an intestinal perfusion technique for the exact measurement of lactose digestion – are laborious. The most widely-used indirect tests are the traditional lactose tolerance test (measurement of serum glucose), the lactose tolerance test with ethanol (measurement of serum galactose), the hydrogen breath test and the urinary galactose test. Genotyping for the C/C_{-13910} variant of lactase persistence/nonpersistence is a new way of determining susceptibility to adult-type hypolactasia; however, it cannot be used as a diagnostic tool to determine lactose intolerance, as the age of reduction of lactase varies (Enattah et al. 2002, Kuokkanen et al. 2003, Rasinperä et al. 2004).

3.4 Processing of milk and its potential gastrointestinal effects

Self-diagnosed cow's milk-related symptoms are commonly reported in questionnaires and interviews (Pelto et al. 1999, Haapalahti et al. 2004, Kokkonen et al. 2004). Some individuals claim to be intolerant to cow's milk, even though neither lactose intolerance nor CMA can be diagnosed. In Nordic countries, a number of consumers claim that they tolerate raw untreated cow's milk and unhomogenised pasteurised cow's milk but show reactions of intolerance to homogenised and pasteurised commercial cow's milk and other dairy products. The parents of certain children who are allergic to cow's milk report the same phenomenon.

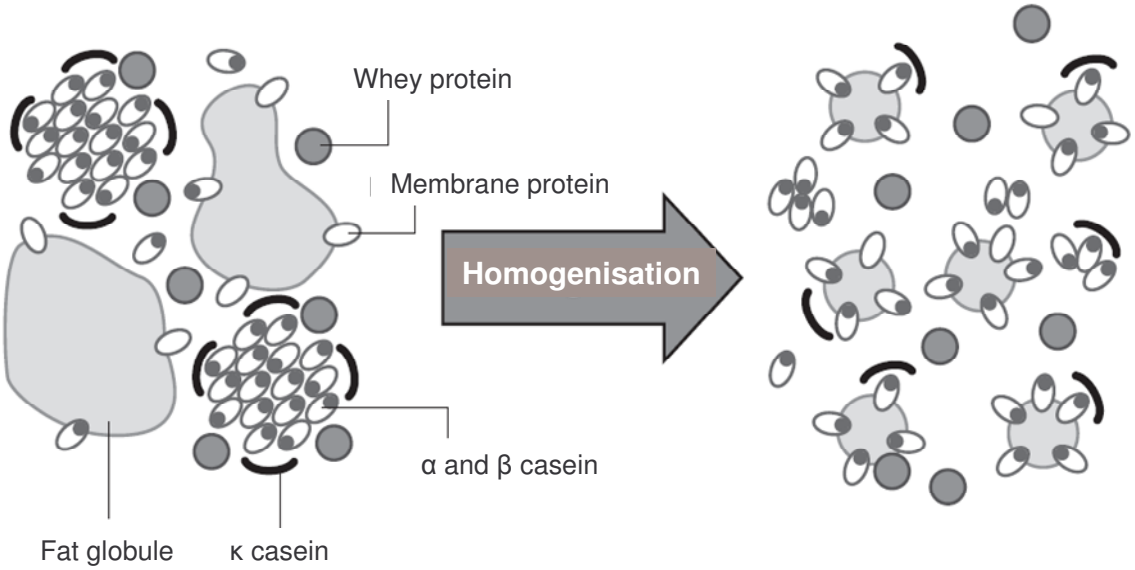

Figure 6 Diagrammatic representation of milk homogenisation: large fat globules divide into smaller droplets, and proteins spread from micelles to cover the new fat droplets.

The hypothesis that might explain how milk homogenisation could cause symptoms is based on the fact that the composition of milk proteins is changed during homogenisation (Fig. 6). Raw cow's milk contains lipid globules of different sizes (Ø 0.1 - 15 µm), which are covered by a phospholipid layer and by membrane proteins. In raw milk, about 80% of the milk proteins are grouped in micelles. During homogenisation large lipid globules are broken up into smaller globules (Ø < 2 µm). As a consequence, the surface area of the milk lipid globules expands and the membrane layers are no longer able to cover the fat globules and are thus partially replaced by milk proteins. In raw milk the majority of the antigenic determinants are inside the casein micelles, but in homogenised milk the concentration of surface-exposed antigenic determinants is higher (Poulsen et al. 1987). This change should not be of physiological significance, since the concentration of antigenic determinants in raw milk is high enough to produce reactions in people with cow's milk protein allergy and antigenic determinants are not harmful to non-allergic people.

Milk is heat-processed to destroy potential pathogens (Jensen 1995). Pasteurisation is a mild heating process (72°C, 15 seconds), and has a minimum effect on the structure and nutritional value of milk, but extends milk preservation time markedly. The ultra high temperature process is a strong heating process (140-150°C, 2 s), and it frees the milk of microbes. Pasteurisation at a high temperature (125-130°C, 0.5-2 s) is a new process for extending the milk's preservation time, called the extended shelf life process, with a minimum effect on the organoleptic characteristics of milk. Heating processes have no effect on lactose composition, nor does heating milk remove or destroy the allergenic properties of milk protein. If one is considering using raw milk, one should take into account the fact that the microbial quality of raw milk may vary. Because of hygiene requirements, the selling of raw milk is limited in the European Union (Council Directive 92/46/EEC).

There is some evidence from animal experiments that the processing of milk, i.e. homogenisation and pasteurisation, causes hypersensitivity reactions in study animals. In animal experiments, homogenised and pasteurised cow's milk given orally to cow's milk-sensitised mice induced anaphylactic shocks (Poulsen et al. 1987), increased IgE production (Nielsen et al. 1989), increased the production of milk-specific immunoglobulins, increased the mass of the gut segment and induced degranulation of mast-cells (Poulsen et al. 1990), while unhomogenised pasteurised cow's milk and unprocessed raw milk induced remarkably fewer symptoms and immunological responses, or none at all.

However, there is no evidence that homogenisation of milk could cause more pronounced milk hypersensitivity in humans than unhomogenised milk. In clinical studies, no difference in tolerance between homogenised and unhomogenised cow's milk has been observed, either in children

with CMA (Hansen et al. 1987, Høst et al. 1988) or in adults with lactose intolerance or milk hypersensitivity (Pelto et al. 2000). In a study by Høst et al. (1990) the maternal intake of homogenised and unhomogenised milk did not affect the passage of bovine β-lactoglobulin to breast milk in either atopic or non-atopic mothers.

Some individuals who experience subjective milk-related gastrointestinal symptoms may actually be sensitive to substances in the diet other than lactose or cow's milk protein. Some Finns report experiencing gastrointestinal symptoms comparable to those of lactose intolerance after consumption of Finnish milk, but abroad they are able to consume local dairy products without symptoms (Paajanen et al. 2004). The most commonly-used milk in Finland is homogenised, pasteurised low-fat milk (0.1-1.5% fat) (Männistö et al. 2003). As far as is known, no differences exist in the texture or processing of milk between Finland and other developed countries, which could explain the dissimilarity of symptoms. However, differences in the diet are likely to occur. The Finnish diet contains marked amounts of indigestible carbohydrates; for example, the average daily consumption of rye products high in indigestible fibre is 100 g in men and 66 g in women (Männistö et al. 2003). Indigestible carbohydrates, such as sweeteners (Born et al. 1994, Storey et al. 2002), fructo-oligosaccharides (Briet et al. 1995, Teuri et al. 1999) and galacto-oligosaccharides (Teuri et al. 1998), have been found to induce symptoms similar to those of lactose intolerance in some individuals, though not in all (van Dokkum et al. 1999, Moore et al. 2003). According to Teuri et al. (1999), so-called pseudohypolactasic subjects mistakenly believe they have lactose intolerance, but are actually reacting to indigestible carbohydrates. We have studied the tolerance of indigestible carbohydrates in adults reporting better tolerance of milk abroad than in Finland, and have concluded that some individuals who report milk-related gastrointestinal symptoms may, in fact, be reacting to indigestible carbohydrates in the diet (Paajanen et al. 2004).

The cause of gastrointestinal symptoms is often difficult to identify, and therefore diet restrictions should be conducted only after a thorough dietary and symptom follow-up.

AIMS OF THE STUDY

The aim of this thesis was to study the effect of cow's milk and its processing on symptoms and immune responses of delayed-type cow's milk hypersensitivity. The specific aims were:

- To study the effect of cow's milk homogenisation on the symptoms of cow's milk-intolerant adults (**I**, **II)** and on the production of cow's milk protein-specific antibodies in healthy adults (**III**)
- To study intestinal immune activation in delayed-type CMA in children (**IV**, **V**)
- To study the occurrence of milk-related reactions and subjective symptoms in relation to verified milk hypersensitivity in young adults (**VI**)

SUBJECTS AND METHODS

1 SUBJECTS

A summary of the study subjects is shown in Table 3.

Studies I, II and III: Study **I** comprised 44 lactose-tolerant adults who had repeatedly experienced better tolerance of unhomogenised than homogenised milk. In Study **II,** 87 adults, and 22 children or the parents of these children, with subjective experience of a better tolerance of unprocessed milk than of processed, were interviewed. The first clinical part of Study **II** comprised 15 lactose-intolerant adults, and the second, clinical part, 35 lactose-intolerant adults. The intolerant subjects were recruited from our previous milk intolerance studies, from consumers who contacted Valio Ltd Consumer Service, and from supermarkets (**I**, **II**). Study **III** comprised 36 adults with good tolerance of milk and a habit of daily milk consumption.

Studies IV and V: The children in Studies **IV** and **V** had been referred to Oulu University Hospital, Finland, for a paediatric gastroenterological consultation because of recurrent gastrointestinal complaints. Study **IV** was made up of a heterogeneous group of 59 children with varying degrees of symptom density, whereas Study **V** comprised 26 children with unmistakable major symptoms. Children with negative cow's milk and cereal challenges and normal endoscopic findings served as controls, and children with untreated celiac disease as disease controls for the children with delayed-type CMA.

Study VI: In Study **VI**, a subgroup of a cohort of healthy children living in northern Finland, who had been gathered in 1994 for a study of risk factors for type 1 diabetes (n=3652, median age 12 y, range 7–16 y) was studied (Kulmala et al. 2001). Those subjects who had been drawn from four rural communities and were 16-21 years old were included in Study **VI** in 2002, making a total of 1078 young adults. Of these, 26 were excluded because of biopsy-proven celiac disease (n=17), suspected celiac disease on the basis of elevated tissue transglutaminase (tTG) (n=7, 3 refused biopsy confirmation, 4 were diagnosed as being healthy) or type 1 diabetes (n=2) (Kulmala et al. 2001, Mäki et al. 2003). The address of 42 subjects was unknown, and therefore a postal questionnaire was mailed to 1010 young adults. In all, 827 subjects (82%) completed the form and made up the final study group. 86 subjects (10%, 11 male, 75 female) reported severe abdominal complaints (severe pain, persistent pain and/or pain combined with abnormal defecation functions); 49 of

these (4 male, 45 female) agreed to take part in a clinical examination and were examined by a paediatric gastroenterologist. Of those interviewed, 149 subjects (18%, 91 male, 58 female) had no history of gastrointestinal or allergic complaints and consumed milk daily, and 27 of these (12 male, 15 female) were included in the study as healthy controls. Additional control subjects (n=10, 2 male and 8 female, median age 16 y, range 14-18 y) were enrolled from outside the original study population, from among patients of Oulu University Hospital, to serve as controls for the endoscopic examination and the measurement of mucosal mRNA expression and intraepithelial T cell densities. The endoscopy controls were studied for prolonged gastrointestinal symptoms but had normal endoscopic findings and remained without any definite diagnosis.

Table 3 Characteristics of the study subjects.

Publication	n	Gender female/male	Age, y mean (range)	Description of study groups
I	44	30/14	38 (18–64)	Better tolerance of unhomogenised than homogenised milk
II				
Interview	22 87	5/17 60/27	5 (1–16) 40 (18–75)	Better tolerance of unhomogenised than homogenised milk
Intervention A	15	13/2	38 (25–62)	Lactose-intolerant subjects
Intervention B	35	32/3	30 (19–57)	Lactose-intolerant subjects
III	36	28/8	40 (19–62)	Good tolerance of milk
IV	59	31/28	10 (1–18)	Delayed CMA (n=31), CD (n=14), controls (n=14)
V	26	18/8	10 (3–15)	Delayed CMA (n=10), CD (n=6), controls (n=10)
VI				
Interview	827	460/367	18 (16–21)	Major GI complaints (n=86), minor GI complaints (n=316), other symptoms (n=276), no symptoms (n=149)
Clinical examination	76	60/16	18 (16–21)	GI symptoms (n=49), controls (n=27)
Endoscopy	22	18/4	17 (14–21)	GI symptoms (n=12), controls (n=10)

CD, celiac disease; CMA, cow's milk allergy; GI, gastrointestinal

2 STUDY DESIGNS

Studies I, II and III: In Studies **I** and **II**, the tolerance of different study milks was compared in randomised, double-blind, cross-over studies (Fig. 7). In Study **I**, two study milks were consumed,

each 2x200ml/d for five consecutive days: 1) pasteurised, unhomogenised milk (1.5% fat), and 2) pasteurised, homogenised milk (1.5% fat) with 20 ml/l of whipping cream added for blinding.

In the first clinical part of Study **II**, three study milks were consumed, each 2 x 200ml/d, for four consecutive days: 1) unpasteurised, unhomogenised organically-produced full-fat milk, 2) pasteurised, homogenised full-fat milk, and 3) pasteurised, homogenised fat-free milk. In the second part of Study **II**, two study milks were consumed, each 2 x 200ml/d, for two consecutive days: 1) unpasteurised, unhomogenised organically produced full-fat milk, and 2) pasteurised, homogenised full-fat milk. In Study **II**, a further 109 subjects were interviewed.

In Study **III**, subjects were challenged for 28 days with both homogenised and unhomogenised cow's milk and dairy products in a randomised, open, cross-over setting (>400 ml of milk daily).

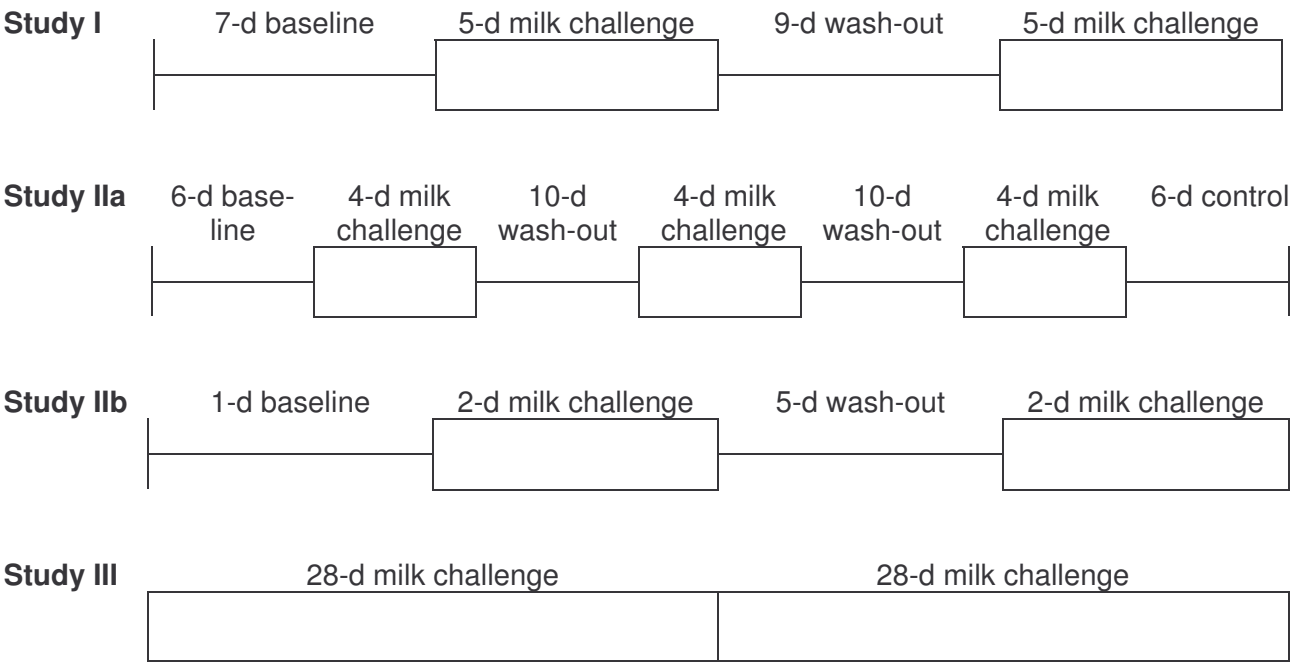

Figure 7 Study designs of Studies **I**, **II** and **III**.

Studies IV and V: In Study **IV**, the 59 patients underwent a gastroduodenoscopy performed by a paediatric gastroenterologist (Fig. 8). The cytokine release (IFN-γ, TNF-α, TGF-β, IL-2, IL-4, IL-5, IL-6 and IL-10), histology and distribution of intraepithelial T cells were examined from intestinal biopsy specimens.

In Study **V**, six biopsies were taken from the bulb of the duodenum and six from the terminal ileum of ten children with delayed-type CMA and ten controls, and six duodenal biopsies were taken from six subjects with celiac disease. The cytokine mRNA expression (IFN-γ, TGF-β, chemokine receptor CC (CCR-4), CCR-5, IL-2, IL-6, IL-10, IL-12p35, IL-12p40 and IL-18), histology and distribution of intraepithelial T cells were examined from the biopsy samples.

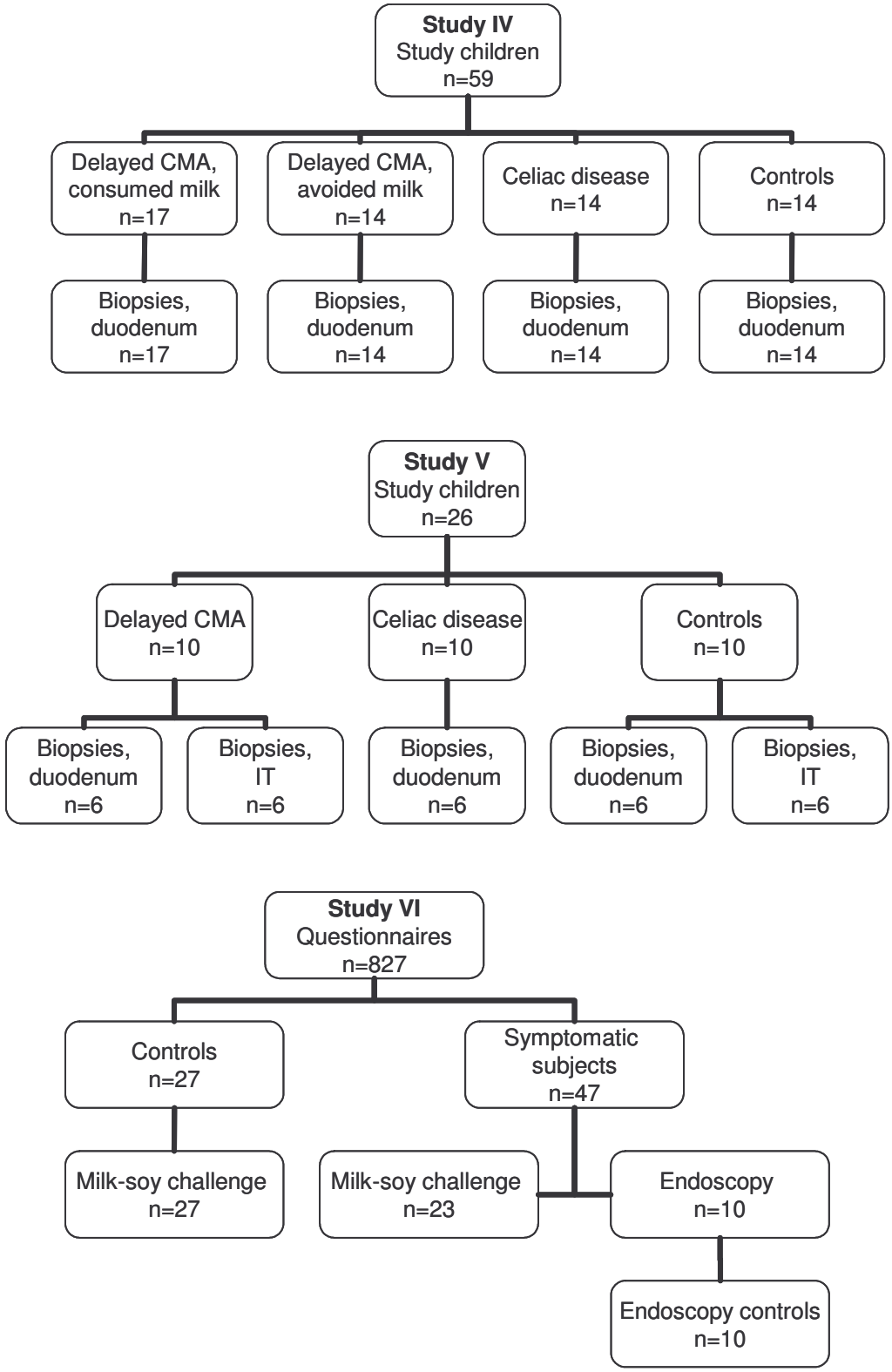

Figure 8 Study designs of Studies **IV**, **V** and **VI**. CMA, cow's milk allergy; IT, ileum terminale

Study VI: Study **VI** consisted of an interview, a food-use survey, a lactose maldigestion test, the genetic test of the C/T_{-13910} variant associated with adult-type hypolactasia, a blinded milk-protein tolerance test, the measurement of antibodies to *Helicobacter pylori*, tTG and milk proteins, as

well as quantification of the plasma concentrations of soluble sICAM-1, and granzymes A and B, and in subjects with severe symptoms, an endoscopic examination and measurement of the mucosal mRNA expression of the following cytokines: IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-10.

3 METHODS

3.1 Questionnaires

Subjects were interviewed about the presence of atopic diseases in each study (**I**-**VI**). The diagnoses of atopic manifestations (asthma, atopic dermatitis and allergic rhinitis) were based on general criteria and accepted only if made by a physician.

The whole study population of Study **VI** was asked about the intensity and frequency of various gastrointestinal symptoms, whether or not the symptoms were related to specific foods, about any atopic history, lactose intolerance and chronic diseases, and the use of milk and dairy products and other foods often related to gastrointestinal symptoms. Gastrointestinal symptoms were enquired about according to Rome II criteria for irritable bowel syndrome (Thompson et al. 1999).

Both the symptomatic subjects and the controls in Study **VI** completed a food frequency questionnaire, concentrating on dairy product consumption and calcium intake, modified from a form validated by Dr. Sulin Cheng and Dr. Arja Lyytikäinen et al. (Calex-study, Jyväskylä, Finland, unpublished data 1998).

In Study **VI**, the parents of the symptomatic subjects and of the controls completed a semi-structured form, also used in a former study (Kokkonen et al. 2004), which included questions regarding the first years of life of the study children, such as the length of time of breast feeding and whether food hypersensitivities, excessive crying, vomiting/regurgitation, or any other gastrointestinal symptoms or allergic symptoms were manifested. Any gastrointestinal and allergic diseases of the parents were also enquired about.

The questionnaires used were pre-tested for clarity on volunteers who belonged to the same age group as the study subjects.

3.2 Investigation of adverse reactions to cow's milk

Assessment of symptoms related to homogenisation of cow's milk

In Study **II**, symptoms related to the ingestion of homogenised and unhomogenised milk were retrospectively enquired about by means of a semi-structured form. In Studies **I** and **II**, the symptomatic response to unhomogenised and homogenised milk was investigated. During the blinded

challenges with the study milks and also during the breaks, the subjects kept a daily record of the consistency and number of their bowel movements, abdominal symptoms, compliance with the milk-free diet, and study drink consumption.

In Study **I**, the subjects ranked daily each gastrointestinal symptom from no symptoms (0) to very severe symptoms (3); the possible range of symptoms was 0-60 points over the 5-day challenge. In Study **II**, the subjects indicated the severity of gastrointestinal symptoms and hardness of faeces, graded from no symptom / very hard faeces (0 mm) to very severe symptoms/very loose faeces (100 mm), by indicating each symptom on a 100 mm-long straight visual analogue scale line; the possible daily range of the sum of symptoms was 0-300 mm in part **IIa**, and 0-400 mm in part **IIb**.

Assessment of symptoms related to cow's milk protein

Cow's milk protein hypersensitivity was determined in Studies **IV**-**VI**. In Studies **IV** and **V**, the diagnosis of hypersensitivity to cow's milk was based on the disappearance of symptoms during the cow's milk elimination diet (4 weeks) and their reappearance in an open challenge test, which was to continue for another 4 weeks with low lactose cow's milk and dairy products if symptoms did not reappear earlier. Those children who were suspected of reacting to both cow's milk and cereals also took part in an open cereal challenge in which the diagnosis of hypersensitivity to cereals was based on the disappearance of symptoms during the cereal elimination diet (4 weeks) and their reappearance in an open challenge test, which was to continue for another 4 weeks with water-based porridge and wheat bread if symptoms did not reappear earlier. As part of the clinical treatment of the study children, either the children or their parents filled in daily a questionnaire on symptoms (abdominal pain, diarrhoea, loose mucous stools and dermatitis) and on milk ingestion during the open milk elimination-challenge procedure. Because the patients were late-onset responders, the gold standard, a blinded challenge, was considered too laborious for clinical work.

In Study **VI** the milk protein tolerance of symptomatic subjects was investigated in a double-blind placebo-controlled food challenge consisting of a 3-week placebo soy drink challenge, a 3-week milk protein challenge blinded with the same soy drink, in randomised order, and a 1-week wash-out period preceding each drink challenge. The subjects were on a milk-free diet during the eight weeks of the placebo-controlled food challenge. They kept a record of the intensity of various gastrointestinal symptoms graded from no symptoms (0) to very severe symptoms (4), of compliance with the milk-free diet, and consumption of the study drink during the 8-week double-blind place-controlled food challenge. The possible daily range of the sum of symptoms was 0-20 points.

Lactose maldigestion

The methods used for the assessment of lactose maldigestion and hypolactasia are summarised in Table 4. Subjects with hypolactasia were diagnosed as lactose intolerant according to their symptomatic responses, monitored by written symptom records (**I**, **II**, **VI**).

Table 4 Methods used for assessment of lactose maldigestion and hypolactasia.

Method	Original publication
Lactose maldigestion tests	
Hydrogen breath test	I, II
Methane breath tests	II
Alcohol-galactose test	VI
Conventional glucose test	IV-VI
Symptom records	I, II, VI
Test for hypolactasia	
Genetic test of C/T_{-13910}	VI

In Study **I**, the digestion of lactose was determined by a 3-hour hydrogen breath test. After an overnight fast, the subjects ingested 25 g of lactose dissolved in 300 ml of water and flavoured with 1 ml of unsweetened juice, and breath hydrogen was measured twice an hour with a Micro H2 Meter (Micro Medical, Kent, UK). In Study **II**, after an overnight fast, the subjects ingested 50 g of lactose dissolved in 250 ml of water and flavoured with 1 ml of unsweetened juice, and breath hydrogen and methane were measured twice an hour for three hours with a Quintron Model DP Microlyzer (Quintron Instrument Co, Milwaukee, WI, USA). An increase in breath hydrogen of $\geq$ 20 ppm was considered hypolactasia (**I**, **II**).

In Study **VI**, an alcohol-galactose test was used (Isokoski et al. 1972), with modifications as reported by Pelto et al. (2000): the subjects ingested 50 g of lactose with 150 mg/body kg of alcohol after an overnight fast, the serum galactose was determined after 40 minutes (Galac, Roche Diagnostics, Basel, Switzerland), and a serum galactose level of less than 0.2 mmol/l was considered hypolactasia. The lactose absorption of seven subjects in Study **VI** whose religious beliefs forbade the consumption of alcohol, as well as the patients of Studies **IV** and **V** who were suspected of being lactose intolerant, was determined after an overnight fast by serial (0, 20, 40 and 60 min) glucose measurements (Glucose HK Liquid, Roche Diagnostics) following a 50 g lactose load. A difference of 1.1 mmol/l or less between the lowest and the highest measurements was considered hypolactasia. In addition, in Study **VI** the subjects were genotyped for the C/C_{-13910}

variant of lactase persistence/nonpersistence (adult-type hypolactasia) (Enattah et al. 2002, Kuokkanen et al. 2003, Rasinperä et al. 2004).

3.3 Immunological investigations

The immunological investigations carried out in this thesis are summarised in Table 5. The subjects of Study **VI** were previously analysed for the HLA-DQ2 molecule encoding genes in order to examine susceptibility to celiac disease and other autoimmune diseases (Mäki et al. 2003), and these data were utilised in Study **VI**. Samples were analysed, by using a screening test, for selected HLA-DQB1 alleles, including DQB1*02 and DQB1*0302, and samples that were positive for the HLA-DQB1*02 allele were further analysed for the presence of associated alleles, HLA-DQA1*0201 and DQA1*05.

Table 5 Immunological investigations.

Method	**Publication**
Antibody measurements	
ELISA: Cow's milk protein specific antibodies	III, VI
ELISA: specific IgA antibodies against tTG	IV-VI
Enzyme immunoassay: specific IgG antibodies against *H. pylori*	VI
Local immunological measurements from biopsy specimens/biopsy supernatants	
ELISA: TGF-β and IL-6	IV
Cytometric bead array: IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-10	IV
rt-PCR: IFN-γ, TGF-β, CCR-4, CCR-5, IL-2, IL-6, IL-10, IL-12p35, IL-12p40 and IL-18	V
rt-PCR: IFN-γ, TGF-β, IL-6, IL-12p35 and IL-18	VI
Systemic immunological measurements from plasma	
ELISA: sICAM-1, granzymes A and B	VI
Cytometric bead array: IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-10	VI
Endoscopic examination and assessment of biopsy specimens	
Assessment of lymphoid nodularity	IV-VI
Histology, villus atrophy	IV-VI
Densities of intraepithelial lymphocytes	IV-VI

ELISA, enzyme linked immunosorbent assay; CCR, chemokine receptor CC; *H. pylori*, *Helicobacter pylori*; IFN-γ, interferon γ; Ig, immunoglobulin; IL, interleukin; rt-PCR, real-time polymerase chain reaction; sICAM-1, soluble intercellular adhesion molecule 1; TGF-β, transforming growth factor β; tTG, tissue transglutaminase; TNF-α, tumor necrosis factor α

Antibody measurements

In Study **III**, the presence of casein-, β-lactoglobulin- and bovine insulin-specific IgG and IgA, and casein-specific IgE, and in Study **VI**, the presence of casein and β-lactoglobulin-specific IgG and IgA in plasma was measured by ELISA assays. Microplates were coated with various antigens at a concentration of 1 µg/ml of buffer (0.2% human serum albumin - phosphate-buffered saline + 0.05% Tween), and blocked with 1% human serum albumin. 100 µl of diluted (from 1:10 to 1:50) samples were pipetted into the wells. The IgG antibodies present were bound with diluted rabbit anti-human IgG (Jackson Immuno Research Inc, West Grove, PA, USA), and the IgA and IgE antibodies, by using biotinylated goat anti-human IgA or IgE (Vector Laboratories Inc, Buringame, CA, USA) and AP Streptavidin (Zymed laboratories, Carlsbad, CA, USA). The reaction was developed by adding phosphate substrate tablets. Optical densities were read at 405 nm for 0.1 s with a multilabel counter (1420 Victor^2, Wallac, Turku, Finland). The results were expressed as optical density units, and these units were considered the relative amounts of the antibodies. All reagents, other than those mentioned above, were from Sigma-Aldrich Co, St. Louis, MO, USA.

In Studies **IV-VI**, serum IgA-class antibodies to tTG were measured by an ELISA method, and in Study **VI** serum IgG-class antibodies to *Helicobacter pylori* by an enzyme immunoassay method (Pyloriset EIA-G III, Orion Diagnostica, Espoo, Finland).

Measurement of cytokines, chemokine receptors and granzymes

In Studies **IV** and **VI**, commercial ELISA kits were used for the analysis of TGF-β1 (Quantikine®, R&D Systems Inc, Minneapolis, MN, USA, Study **IV**), IL-6 (PeliKine Compact™, Central Laboratory of the Netherlands Red Cross, Amsterdam, Netherlands, Study **IV**), sICAM-1 (HyCult Biotechnology, Uden, Netherlands, Study **VI**) and granzymes A and B (Sanquin, Amsterdam, Netherlands, Study **VI**), and cytometric bead array human Th1/Th2 kit (BD Biosciences, San Diego, CA, USA) for the analysis of certain cytokines (**IV**, **VI**).

In Studies **V** and **VI**, the expression of certain cytokines and chemokine receptors was measured by real-time quantitative reverse transcriptase polymerase chain reaction (rt-PCR). Total RNA was extracted from frozen biopsies by RNA Total Gen Elute Mammalian RNA kit (Sigma-Aldrich Co). Reverse transcription reaction was carried out by using TaqMan Reverse Transcription Reagents (all rt-PCR reagents from Applied Biosystems, Foster City, CA, USA). The PCR was performed in triplicate wells using TaqMan pre-developed assay reagent universal MasterMix, 1 x pre-developed assay reagent primers/probes, and template cDNA, and measured with an automated fluorometer, the ABI-Prism 7700 Sequence Detection System (Applied Biosystems). Ribosomal 18S was used as an endogenous control. The expression of each cytokine was also measured

from a home-made calibrator sample, which was prepared from the phytohemagglutinin-stimulated peripheral blood mononuclear cells of a healthy subject. The comparative threshold method was used to quantitate the gene transcription in the samples. To obtain the whole numbers for plots, the relative numbers were multiplied by 100, excluding IL-18, whose numbers were not multiplied.

Endoscopic examination and assessment of biopsy specimens

The subjects of Studies **IV**, **V** and **VI** who had persistent gastrointestinal symptoms were studied by endoscopy. Endoscopies were carried out before any food challenges. Gastro-duodenoscopies were performed with an Olympus CIF-IT140 (Olympus, Tokyo, Japan), and colonoscopies with an Olympus GF Q1401 (Olympus), both under general anaesthesia.

The assessment of lymphoid nodularity on the mucosa of the duodenum and/or ileum was based on endoscopic evaluation after filling the area to be inspected with air. Only a cluster of lymphoid nodules (n>10 nodules) was considered significant. The criteria used to assess the severity of LNH were: grade 0, no lymphoid follicles present; grade 1, mild to moderate LNH, small lymphoid follicles dispersed on the walls; and grade 2, severe LNH, massed with lymphoid tissue.

For histopathology, the biopsy specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. The lengths of crypt and villi were measured with an ocular micrometer.

For immunohistochemical stainings, biopsy samples were stained, and the numbers of lymphocytes were counted per mm of epithelium under a light microscope with a 100x flat field objective. 20-30 fields (1-1.5 mm) were examined for the presence of CD3+ T lymphocytes and αβ and γδ TCR-bearing intraepithelial lymphocytes.

3.4 Statistical analyses

The following analyses were performed to estimate the significance of the differences between the cross-over milk challenges: the analysis of variance (ANOVA) for repeated measurements was used to study treatment, period and carry-over effects (**I–III**); the Wilcoxon test, to compare the intensity of the symptoms (**II**); the non-parametric McNemar, to compare the prevalence of symptoms between the study challenges (**I**, **II**); and because significant period and carry-over effects were found, Fisher's exact test was also utilised in Study **II**.

Differences between the patient groups were determined with: the one-way ANOVA with a post hoc test (**IV**); the independent samples *t* test (**VI**); and in the case of limited data, the non-

parametric Mann-Whitney U-test and the Kruskall Wallis test (**V**). Dichotomous variables were tested with the Pearson Chi-Square test (**V**, **VI**) and correlations with the Pearson correlation coefficient (**IV**). The 95% confidence intervals (CI_{95}) for the prevalence of symptoms were tested with the normal distribution approximation or, in the case of small sample size (n<50), by the exact method based on binomial distribution (**VI**).

In the cases of skewed data, logarithmic (ln) transformation was used if parametric analyses were performed (**III**, **IV**, **VI**). Depending on the number of the subjects and the skewness of the data, means are presented with SEM (**I**) or with CI_{95} (**VI**), geometric means with CI_{95} (**III**), and medians with ranges (**II**, **IV**, **V**).

Data were analysed using SPSS software (SPSS Inc, Chigago, IL, USA). A p value of <0.05 was considered significant.

4 ETHICS

The study protocols of Studies **I** and **III** were approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa, that of Study **II** by the Ethics Committee of the Foundation for Nutrition Research, and those of Studies **IV**, **V**, and **VI** by the Ethics Committee of Oulu University Hospital. Informed, written consent was obtained from the study subjects (**I**-**III**, **VI**) and/or the parents of the study children (**IV**, **V**).

RESULTS

1 EFFECT OF MILK HOMOGENISATION ON SYMPTOMS AND ON ANTIBODY RESPONSE TO MILK

1.1 Symptoms related to milk homogenisation (I, II)

In an interview, subjectively milk-intolerant subjects mainly reported gastrointestinal symptoms related to the intake of processed milk (**II**). However, in the blinded challenges, the homogenised and unhomogenised milks produced equal symptoms in subjects subjectively sensitive to homogenised milk (**I**) as well as in those who had been diagnosed as lactose intolerant (**II**).

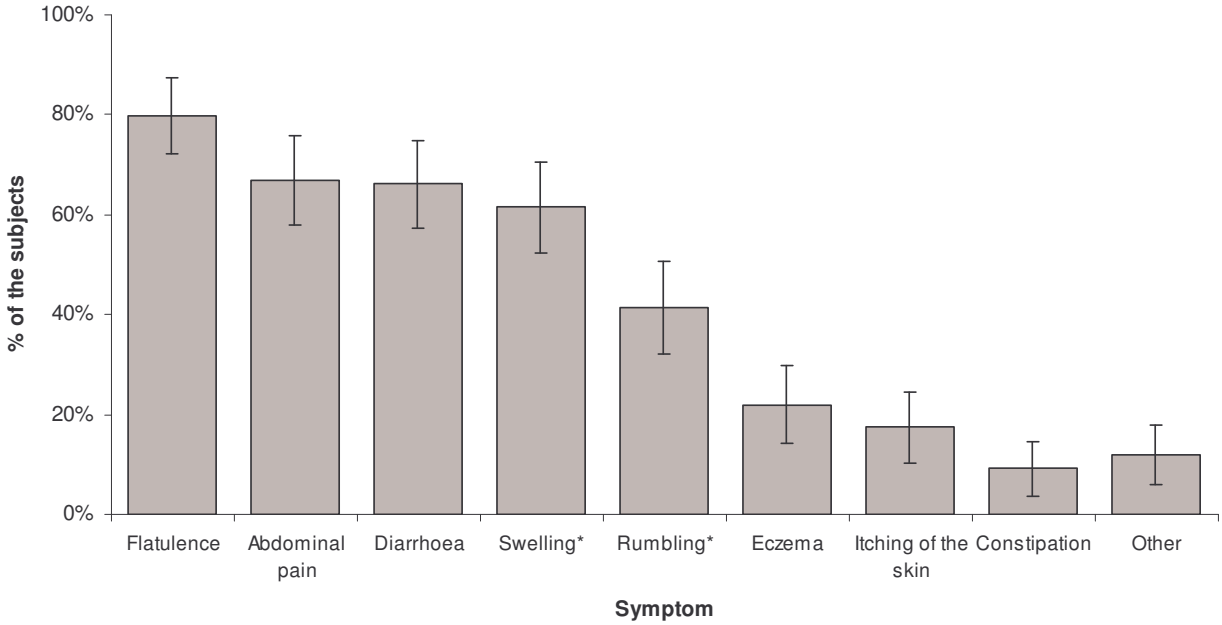

Figure 9 Symptoms reported as being caused by homogenised milk by 109 subjects interviewed, subjectively intolerant to homogenised milk. Symptoms are expressed as percentage of subjects with 95% confidence interval (**II**). *Swelling and rumbling of the stomach.

Questionnaire

The subjectively milk-intolerant subjects interviewed in Study **II** for the most part reported gastrointestinal symptoms related to the intake of processed milk, though skin or other symptoms were occasionally mentioned (Fig. 9). Gastrointestinal symptoms appeared during the 12 h following milk ingestion in all the subjects, while skin symptoms took up to 3 days to appear (Fig. 10A). In

most cases (84% of the subjects) the symptoms disappeared within 24 h of the termination of milk ingestion; skin symptoms were the slowest to fade (Fig. 10B).

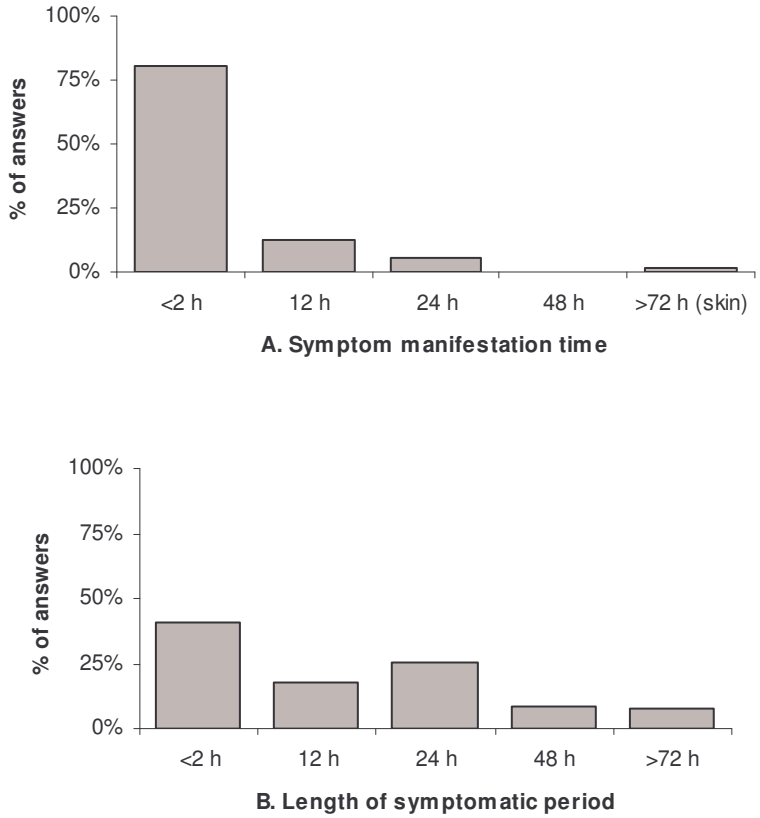

Figure 10 A. Time to symptom manifestation (123 answers), and B. length of symptomatic period after ingestion of homogenised milk (122 answers) as reported by 109 subjects subjectively intolerant to it (**II**). Some subjects reported different times for different symptoms.

Most subjects (81%) reported no symptoms from unhomogenised milk, a few (8%) reported weaker symptoms from unhomogenised than from homogenised milk, and some (7%) reported that unhomogenised milk also occasionally induced symptoms. 41% of the subjects had tried a totally milk-free diet, during which they had experienced no symptoms at all. 26% were diagnosed as suffering from lactose intolerance, 4% irritable bowel syndrome, and 2% celiac disease.

The unhomogenised milk most commonly used by the subjects was a commercial, organically produced, pasteurised, unhomogenised milk (103 subjects); 18 subjects had consumed unpasteurised, unhomogenised farm milk. Unhomogenised milk was drunk without symptoms by 77% of the subjects, while the rest used it only in cooking. Most of the subjects were able to consume certain dairy products without suffering any symptoms (Fig. 11), and many subjects (46%) were able

to consume small amounts of homogenised milk as an ingredient in cooking. Dose-response was mentioned by 76% of the subjects.

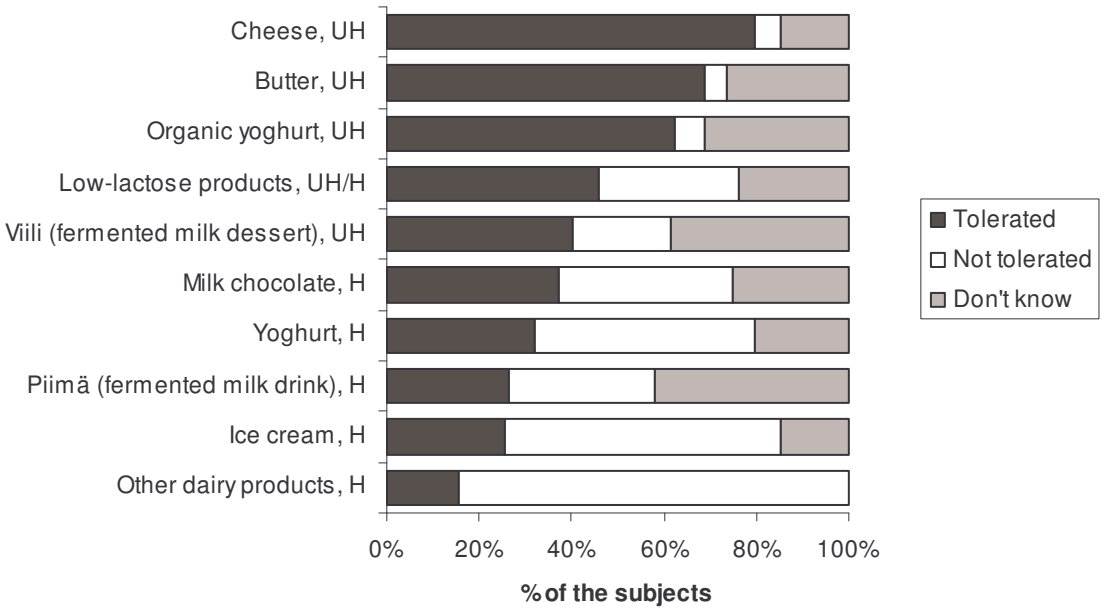

Figure 11 Milk products reported as being tolerated or otherwise by subjects interviewed, who were subjectively intolerant to homogenised milk (n=109, **II**). UH, unhomogenised; H, homogenised

Challenges with unhomogenised and homogenised milk

The symptomatic responses of the subjects were observed during challenges with unhomogenised and homogenised milks (Studies **I** and **II**). There was no difference between the effect of homogenised and unhomogenised milk on the severity or the prevalence of the symptoms in either of these two studies. The symptom scores are summarised in Figure 12. In Study **I**, the difference between the scores of the homogenised and the unhomogenised milk during the 5-day milk challenges was 0.9 points (CI_{95} -2.4–4.2) (p=N.S., theoretical max. symptom score 60 points). In Study **IIa**, based on the period averages, the median sum of gastrointestinal symptoms was 74 mm (range 0–185) for unprocessed milk, 51 mm (9–117) for processed full-fat milk and 90 mm (22–167) for processed fat-free milk (p=N.S. for all, theoretical max. score 300 mm). In Study **IIb**, based on the period averages, the median sum of gastrointestinal symptoms was 60 mm (range 0–280) for unhomogenised and 55 mm (0–290) for homogenised milk (p=N.S., theoretical max. score 300 mm). The median stool characteristics and number of stools remained constant during Studies **I**, **IIa** and **IIb**.

Even though in general unhomogenised milk was not better tolerated, in Studies **I** and **IIb**, two subjects tolerated it markedly better. In Study **I**, one of the two had 40 points more symptoms dur-

ing the unhomogenised than during the homogenised milk period, and the other, 20 points more symptoms, the maximum symptoms per milk period being 60 points. In Study **I**, two subjects gave up homogenised milk and one subject gave up unhomogenised milk, because of severe reactions. In Study **IIb**, two subjects stopped drinking the processed milk after the first test day, because of very loose stools and other severe symptoms.

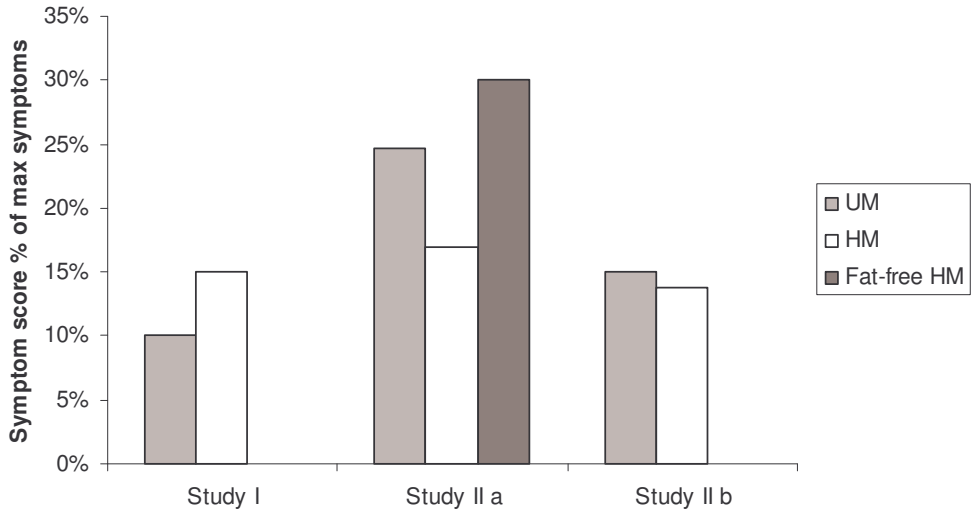

Figure 12 Median symptom scores as percentages of the theoretical maximum symptom score during challenges with unhomogenised milk (UM) and homogenised milk (HM). Study **I** comprised subjectively milk-intolerant subjects (n=44) and Studies **IIa** (n=15) and **IIb** (n=35), lactose-intolerant subjects.

In Studies **I** and **IIb**, the severity and prevalence of symptoms were higher during the milk challenges than during the milk-restricted run-in period. In Study **IIa**, the prevalence of flatulence and abdominal bloating, to at least a moderate degree, was high during all three milk periods, and even during the low-lactose control periods.

In Study **I**, the subjects experienced more symptoms (mean 10.8 points, CI_{95} 8.4–13.2) during the second challenge than during the first (7.0 points, CI_{95} 4.5–9.5), regardless of the order in which they received the different types of milk (period effect p=0.02). However, the sum of symptoms was similar during the run-in period and the wash-out period between the challenges. Some carry-over and period effect also affected the milk periods of Study **IIb**. The subjects experienced more symptoms during the first period (median 85 mm) than during the second (45 mm), independent of which milk was ingested (period effect p=0.002), but the carry-over effect was non-significant (p=0.72). In particular, abdominal pain and bloating were more common during the first milk period (66% and 83% respectively) than during the second milk period (40% and 63%) (period effect for abdominal pain p=0.05 and for bloating p=0.07, and carry-over effect for ab-

dominal pain p=0.05). When the period effect was eliminated by only taking into account the first period, there was a tendency towards a smaller number of symptomatic subjects during the unprocessed compared to the processed milk period (for pain 53% vs. 78%, p=0.16, and for bloating 71% vs. 94%, p=0.09).

1.2 Effect of milk homogenisation on antibody production (III)

In Study **III**, IgG and IgA against casein, β-lactoglobulin and bovine insulin, and IgE against casein were measured by ELISA from serum samples taken from 36 milk-tolerant adults at baseline and at the end of the 28-d-long challenges with homogenised and unhomogenised cow's milk. The antibody production of the subjects remained constant, with no differences between the challenges with the two milks (Fig. 13). Inter-individual variation was notably greater than intra-individual or the variation between the milk challenges, as seen in Figure 14. The order of the milk challenges did not affect the results.

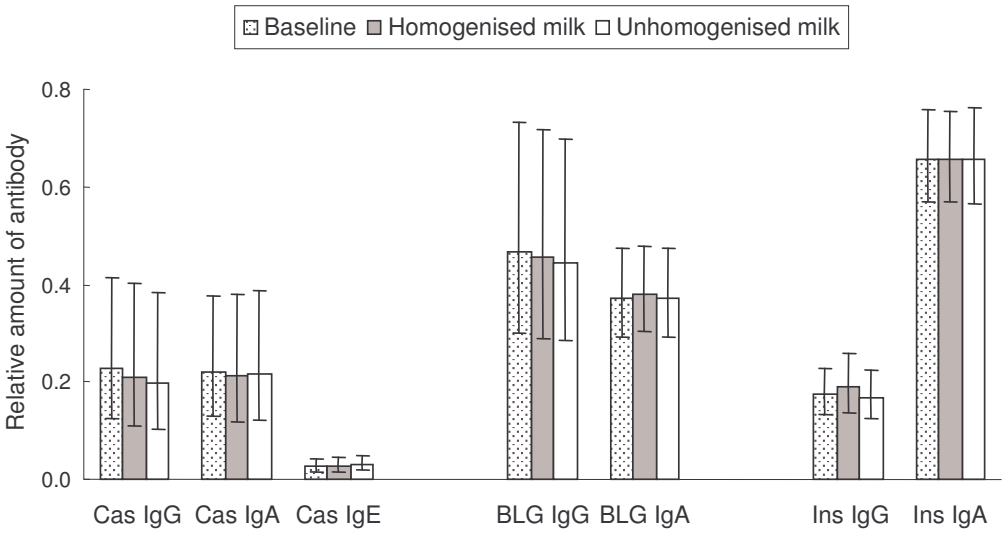

Figure 13 Relative amounts of antibodies studied, at baseline and during the challenges with homogenised and unhomogenised milk (geometric mean ± 95% CI, n=36). Cas, casein; Ig, immunoglobulin; BLG, β-lactoglobulin; Ins, insulin

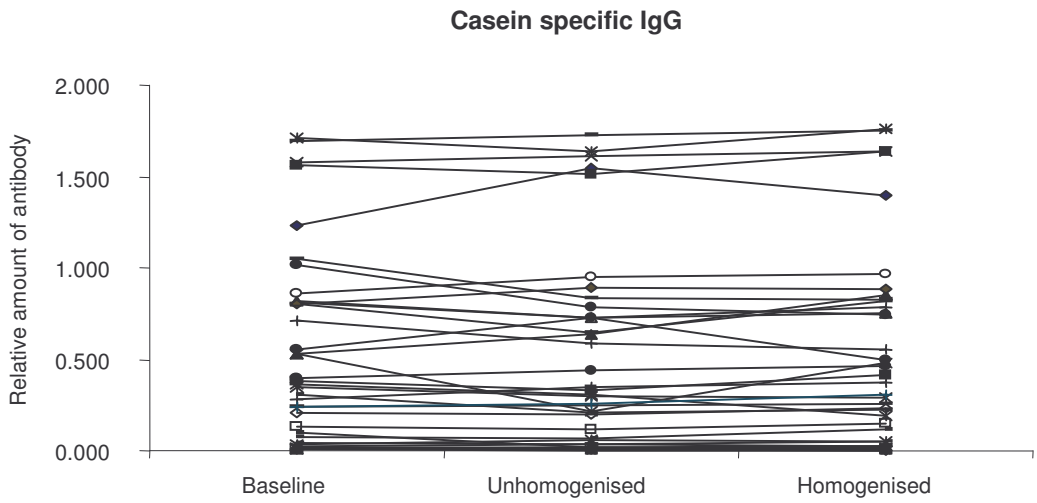

Figure 14 Production of casein-specific immunoglobulin (Ig) G by each study subject, at baseline and after challenges with unhomogenised and homogenised milk, showing that individual antibody production of the subjects remained constant over the challenges (n=36). Each line represents one subject.

2 INTESTINAL IMMUNE ACTIVATION IN DELAYED-TYPE COW'S MILK ALLERGY, AND IN IMMUNE-LIKE GASTROINTESTINAL SYNDROME

2.1 Endoscopic findings, histopathology and intraepithelial lymphocytes (IV-VI)

In Studies **IV** and **V**, lymphonodular hyperplasia of the duodenal bulb or terminal ileum was a characteristic endoscopic finding in most of the children with delayed CMA, though not in all (Table 6). In Study **VI**, none of the young adults with gastrointestinal symptoms or the controls showed lymphonodular hyperplasia. Only the celiac disease cases showed duodenal villous atrophy or crypt hyperplasia.

In the duodenal samples, the densities of intraepithelial T cells were slightly up-regulated in those children with delayed CMA who were exposed to dietary cow's milk (**IV**, **V**), and in young adults with gastrointestinal symptoms (**VI**) (Table 7, Fig. 15). The children with celiac disease had by far the highest densities of $CD3^+$, $\alpha\beta^+$ and $\gamma\delta^+$ T-cells in the duodenum compared to any other group ($p<0.001$ for all, **IV**, **V**), while densities were normal in the controls (**IV-VI**). These results show an up-regulation of duodenal intraepithelial T cells in untreated food hypersensitivity. In fact, in Study **IV**, densities were normal in those children with CMA who avoided cow's milk. Densities of intraepithelial lymphocytes in the terminal ileum are presented in Figure 16.

Table 6 Lymphonodular hyperplasia (LNH) found in the bulb of the duodenum or the terminal ileum in the study groups. Figures represent numbers of LNH subjects out of total group numbers.

	LNH of duodenum	**LNH of ileum**
Study IV		
Delayed CMA	17/33	no samples
Celiac disease	1/17	no samples
Controls	0/14	no samples
Study V		
Delayed CMA	3/6	6/6
Celiac disease	0/6	no samples
Controls	0/6	0/6
Study VI		
GI symptoms	0/8	0/7
Controls	0/8	0/6

CMA, cow's milk allergy; GI, gastrointestinal

Table 7 Up-regulation of intraepithelial lymphocytes in children with delayed cow's milk allergy (CMA, **IV**, **V**) and young adults with immune-like gastrointestinal syndrome (**VI**). P-values compared to the controls are presented as follows: $^{(*)}$p<0.065, *p<0.05, **p<0.01.

	CMA1, **IV**	CMA2, **IV**	CMA1, **V**	GI syndrome, **VI**
Biopsies from duodenum				
$CD3^+$	↑*	↔	↔	↑$^{(*)}$
$\alpha\beta^+$	↑**	↑*	↑$^{(*)}$	↔
$\gamma\delta^+$	↔	↔	↔	↔
$\gamma\delta^+/CD3^+$	↔	↔	↔	↔
Biopsies from ileum				
$CD3^+$	no samples	no samples	↔	↔
$\alpha\beta^+$	no samples	no samples	↔	↔
$\gamma\delta^+$	no samples	no samples	↑*	↔
$\gamma\delta^+/CD3^+$	no samples	no samples	↑$^{(*)}$	↔

↑ increased, ↔ stable
CMA1, children with delayed CMA who consumed cow's milk; CMA2, children with delayed CMA who avoided cow's milk; GI syndrome, young adults with immune-like gastrointestinal syndrome

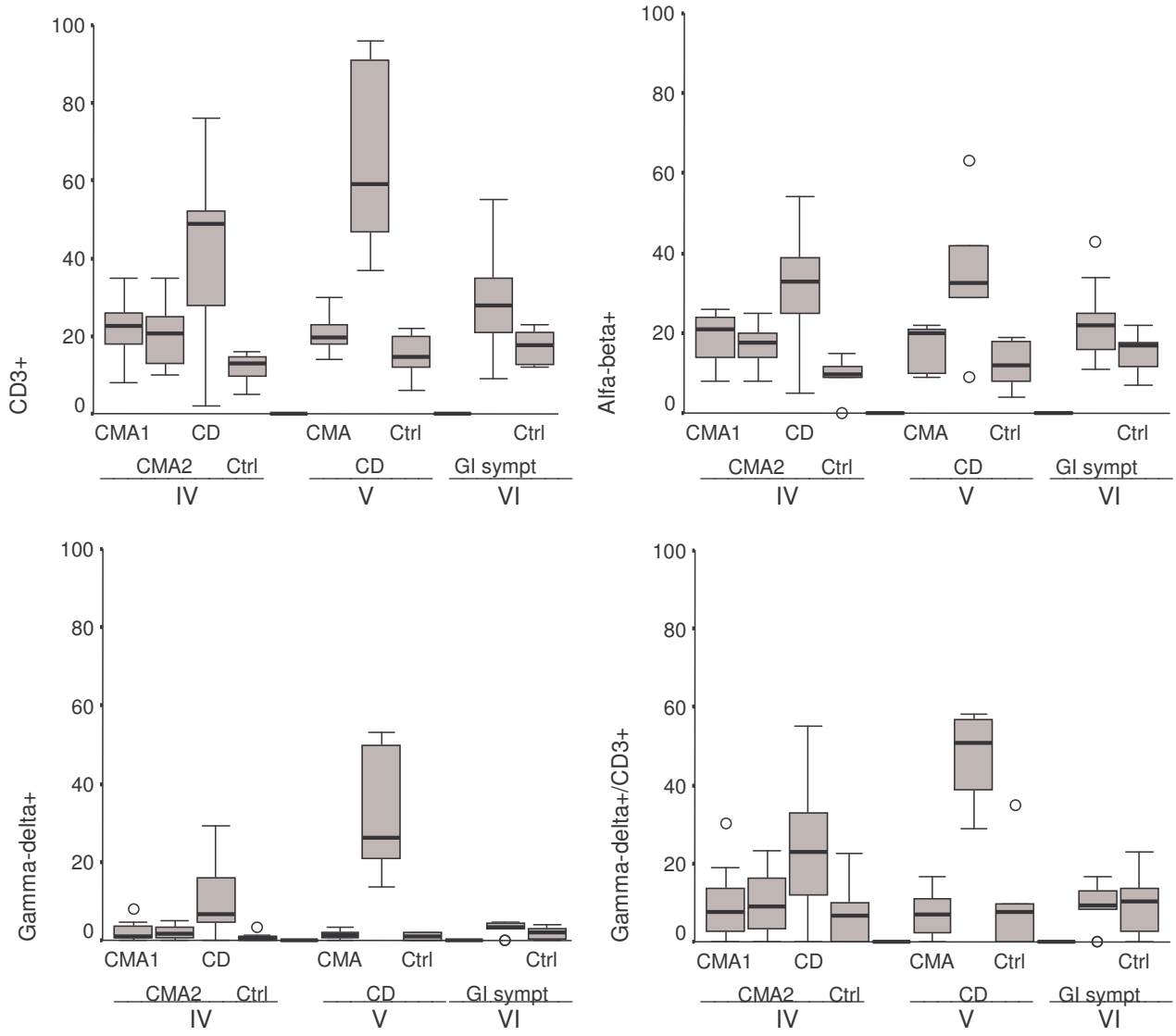

Figure 15 Densities of intraepithelial CD3+, αβ+, γδ+ T cells and γδ+/CD3+ ratio in the bulb of the duodenum, presented as box-plot figures (**IV**-**VI**). Boxes represent the 95% confidence intervals with medians, vertical lines represent ranges, and outliers are marked as circles. The study groups are labelled as follows: Study **IV**: CMA1, children with delayed cow's milk allergy who consumed milk; CMA2, children with delayed CMA who avoided milk; CD, celiac disease; Ctrl, controls. Study **V**: CMA, delayed CMA; CD, celiac disease; Ctrl, controls. Study **VI**: GI sympt, subjects with GI symptoms (immune-like GI syndrome); Ctrl, controls.

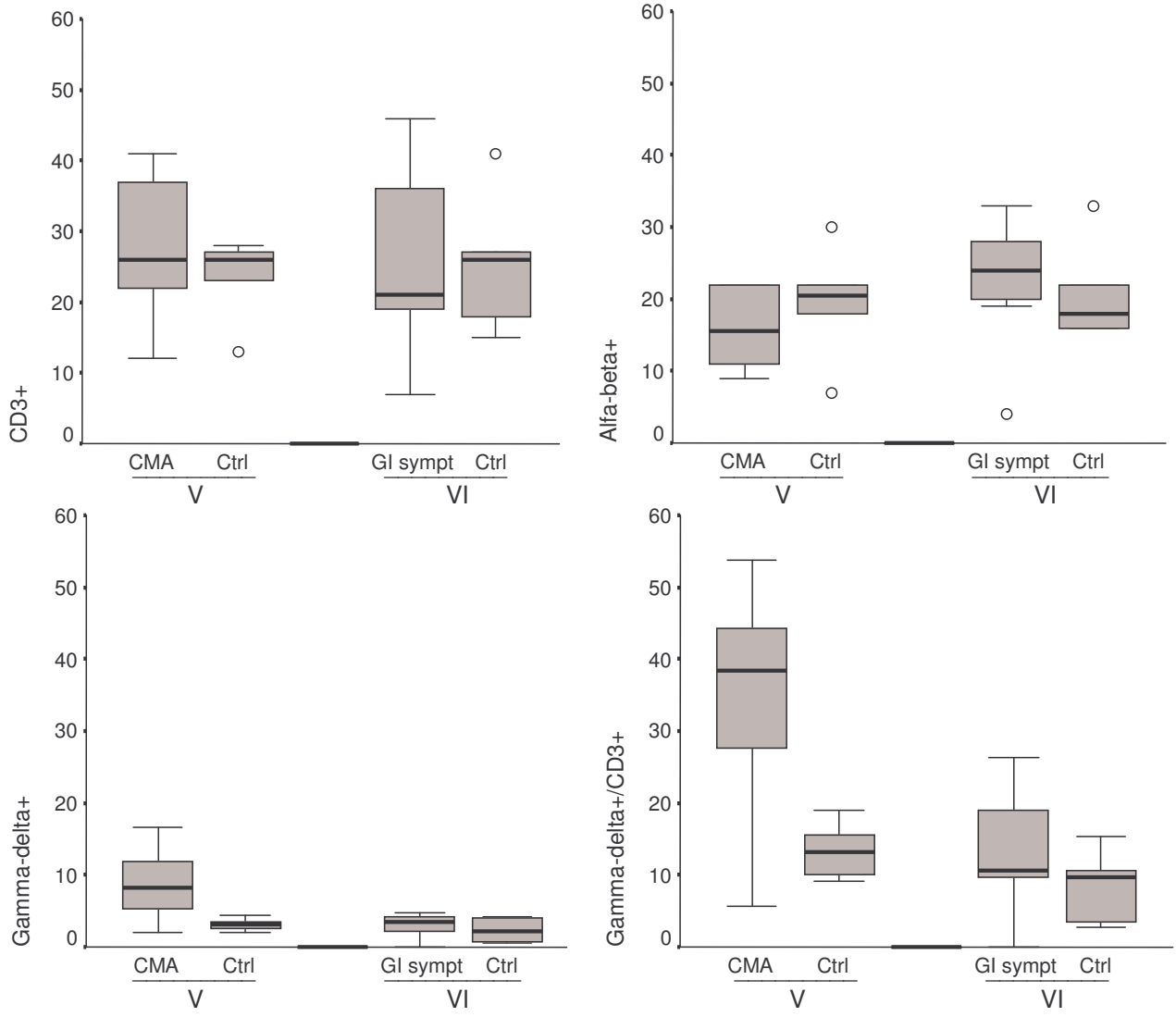

Figure 16 Densities of intraepithelial CD3+, αβ+, γδ+ T cells and γδ+/CD3+ ratio in the terminal ileum, presented as box-plot figures (**V**-**VI**). Boxes represent 95% confidence intervals with medians, vertical lines represent ranges, and outliers are marked as circles. The study groups are labelled as follows: Study **V**: CMA, delayed cow's milk allergy; Ctrl, controls. Study **VI**: GI sympt, subjects with GI symptoms (immune-like GI syndrome); Ctrl, controls.

2.2 Immune profile in delayed-type cow's milk allergy (IV, V)

The intestinal release or mRNA expression of cytokines, chemokine receptors and granzymes in the children with delayed CMA and the young adults with gastrointestinal symptoms are summarised in Table 8. In Study **IV**, the children with delayed CMA secreted more IFN-γ than the controls (median 33.5 vs. 10.0 pg/ml, p=0.004), and more still than the children with celiac disease (33.5 vs. 14.4 pg/ml, p=0.005). The children with delayed CMA who consumed cow's milk

showed lower secretion of TGF-β than those with delayed CMA who avoided cow's milk (399.5 vs. 612.3 pg/ml, p=0.050), a tendency towards lower secretion of TGF-β than the controls (399.5 vs. 441.1 pg/ml, p=0.078), and slightly higher secretion of IL-4 (5.5 vs. 2.7 pg/ml, p=0.016) and IL-10 (4.6 vs. 2.8 pg/ml, p=0.059).

In Study **V**, the children with delayed CMA expressed less IL-2 and IL-18 mRNA in the duodenum (median 0.78 vs. 2.22, p=0.055; 54.28 vs. 239.87, p=0.055), and more CCR-4 and IL-6 mRNA in the terminal ileum (22.39 vs. 9.60, p=0.055; 0.57 vs. 0.12, p=0.016) than the controls. The mRNA expression levels of the regulatory cytokines, TGF-β and IL-10, remained similar in all three groups.

Table 8 Activation of cytokines, chemokine receptors and granzymes in children with delayed CMA (**IV**, **V**) and young adults with immune-like gastrointestinal syndrome (**VI**). P-values compared to the controls are presented as follows: $^{(*)}$p<0.065, *p<0.05, **p<0.01, ***p<0.001.

	CMA1, **IV**	CMA2, **IV**	CMA1, **V**	GI syndrome, **VI**
Biopsies from duodenum				
IFN-γ	↑**	↑*	↔	↔
TGF-β	↓$^{(*)}$	↔	↔	↔
IL-2	↔	↔	↓$^{(*)}$	↔
IL-4	↑*	↔	not studied	not studied
IL-10	↑$^{(*)}$	↔	↔	↔
IL-18	not studied	not studied	↓$^{(*)}$	↔
Biopsies from ileum				
IL-6	no samples	no samples	↑*	↔
CCR-4	no samples	no samples	↑$^{(*)}$	not studied
Blood samples				
sICAM-1	not studied	not studied	not studied	↑**
Granzyme-A	not studied	not studied	not studied	↓***

↑ increased; ↓ decreased; ↔ stable
CMA1, children with delayed cow's milk allergy who consumed cow's milk; CMA2, children with delayed CMA who avoided cow's milk; GI syndrome, young adults with immune-like gastrointestinal syndrome, and; CCR, chemokine receptor CC; IFN-γ, interferon γ; IL, interleukin; sICAM-1, soluble intercellular adhesion molecule 1; TGF-β, transforming growth factor β

2.3 Immune profile in immune-like gastrointestinal syndrome (VI)

In Study **VI**, the symptomatic subjects (n=8) studied by endoscopy tended to express slightly more TGF-β mRNA (3.3 vs. 1.0, p=0.073) and IL-12p35 mRNA (26.5 vs. 13.6, p=0.075) in the duode-

nal biopsy samples than the controls (n=8). The symptomatic subjects (n=47) had higher mean plasma concentrations of sICAM-1 (71.4 vs. 57.8 ng/ml, p=0.008) and lower plasma concentrations of Granzyme A (29.6 vs. 56.0 pg/ml, p=0.001) than the controls (n=27). In systemic immune defence, i.e. cytometric bead array analysis of the plasma cytokine concentrations, no differences between the groups were found.

3 GASTROINTESTINAL DISORDERS IN YOUNG ADULTS

3.1 Gastrointestinal symptoms and diseases in young adults (VI)

In a population-based survey (**VI**), about half the 827 subjects (n=402, 49%) reported having had one or more abdominal complaints during the previous year, in 316 the symptoms being minor and in 86, major. 43 subjects (5.2%) fulfilled the Rome II criteria for irritable bowel syndrome. Gastrointestinal symptoms were far more frequent among the females than among the males: in the group with major gastrointestinal symptoms, 87% (75/86) were female compared to 39% (58/149) in the healthy group ($p<0.001$).

Of the 86 subjects with major gastrointestinal symptoms who were interviewed, 49 agreed to undergo clinical examination. Ten of these (20%) had elevated serum IgE (median 204, range 115-2222 IU/l). One subject was positive for IgA antibodies towards tTG, was diagnosed by gastroscopy as having celiac disease, and excluded from further immunological measurements. One subject was diagnosed as having ulcerative colitis, and was also excluded from further immunological measurements. Of the symptomatic subjects, 3 (6%) were positive for IgG-class *Helicobacter pylori* antibodies as were 3 (11%) of the healthy controls.

3.1 Tolerance of milk in young adults (VI)

In the questionnaires (n=827), 13.1% reported that they suffered from lactose intolerance. In the clinical examination, the lactose maldigestion test was positive in 16 of the 47 symptomatic subjects (34.0%). However, only four (8.5%) had the C/C_{-13910} genotype associated with adult-type hypolactasia and the rest carried the C/T_{-13910} genotype of lactase persistence. Out of 27 symptom-free controls, one (3.7%) had the C/C_{-13910} genotype of adult-type hypolactasia, 15 (55.5%), the C/T_{-13910}, and 11 (40.7%), the T/T_{-13910} genotype of lactase persistence.

The symptomatic subjects had lower concentrations of casein-specific IgG than the controls (n=47 vs. n=27; 0.91vs. 1.20; mean diff. 0.28, CI_{95} 0.01–0.57; p=0.043). The difference was great-

est between those symptomatic subjects who did not drink milk and the controls (n=31 vs. n=27, 0.83 vs. 1.20; mean diff. 0.37, CI_{95} 0.05–0.68; p=0.023).

In the blinded cow's milk-protein challenge, the cow's milk and the placebo soy drink provoked equal symptoms (mean difference -0.34, CI_{95} -7.1–6.4, p=0.920). Of the 23 symptomatic subjects who completed the challenge, 16 experienced intense gastrointestinal symptoms during both the milk and the placebo soy challenges, five experienced more symptoms (>10 points/week) during the 3-week low-lactase milk challenge, and nine during the 3-week placebo soy challenge, while in nine no difference was observed between the challenges. Only two subjects had severe and permanent symptoms during the milk challenge, and one of these was lactose intolerant. Among the 25 non-symptomatic controls who completed the challenge, one experienced some gastrointestinal symptoms during the milk challenge, and one other, during the soy challenge. The theoretical maximum sum of gastrointestinal symptoms was 140 points/week.

In the questionnaires, 109 of the 827 (13.2%, CI_{95} 10.9–15.5%) reported that they did not drink milk – this was more common in the females (86/458, 18.8%) than in the males (23/369, 6.2%, p<0.001), and in the group with major gastrointestinal symptoms (31/86, 36.0%) than in all the other subjects together (78/741, 10.5%, p<0.001). Those with major gastrointestinal complaints reported more food-related symptoms than did all the other subjects (p<0.001), and reported more abdominal pain (n=55, 64%), diarrhoea (n=27, 31%), constipation (n=9, 11%) and skin symptoms (n=3, 4%) related to milk ingestion. Abdominal pain was commonly reported after ingestion of all foods enquired about, including non-dairy products. In the food usage frequency form, it was seen that the symptomatic study subjects restricted their diet in various ways compared to the healthy controls.

None of the symptomatic subjects who were clinically examined reported that they eliminated all dietary cow's milk proteins, either at the time of the interview or previously. However, avoidance of milk and/or dairy products was common, since only 14 (29%) consumed such products without restriction.

DISCUSSION

1 METHODOLOGICAL ASPECTS (I-VI)

Selection of study subjects

Studies I-III: The tolerance of homogenised and unhomogenised/unprocessed cow's milk was compared in two studies, in three different settings, and no differences in the symptoms during the challenges with homogenised and unhomogenised milk were discerned (**I**, **II**). Nor, in Study **III**, did antibody production by milk-tolerant adults differ between the challenges with homogenised and unhomogenised milk. The subjects of Study **I** had subjectively experienced better tolerance of unhomogenised than homogenised milk, and the subjects of Study **II** were lactose-intolerant volunteers. Even though it would have been interesting, it was not possible in Study **III** to study milk-intolerant subjects since they are not able to consume milk long enough to induce alterations in antibody levels, as far as non-IgE-mediated reactions are concerned.

Studies IV-VI: To obtain an objective view of intestinal changes in delayed-type CMA, these were examined in study populations differing from each other (**IV-VI**). In Studies **IV** and **V**, both the subjects and the controls had been referred to Oulu University Hospital for gastroenterological consultation because of recurrent gastrointestinal complaints. In Study **IV**, all those patients diagnosed as having delayed-type CMA or celiac disease were included. On the other hand, only well-defined controls and those with major symptoms of delayed-type CMA or celiac disease were included in Study **V**, and therefore the subjects were more homogeneous than the subjects of the previous study. For Study **VI**, symptomatic young adults were selected for endoscopy from a population-based cohort. Many of them suspected that milk was the cause of their symptoms, but in a blinded milk challenge their symptoms were found not to relate to milk. It is not unexpected that cytokine results and endoscopic findings differed between the study populations since there was distinct symptomatic heterogeneity of the three populations. However, the densities of intraepithelial lymphocytes were up-regulated and found to be fairly similar in all these groups.

In Studies **IV**-**VI**, children with negative challenges to cow's milk and cereal and with normal endoscopic findings served as controls, since the cause of their gastrointestinal complaints were considered to be of psychological origin. As far as children are concerned, the indication for invasive gastro-/colonoscopia has to be clinical, and symptom-free healthy controls cannot be used because of ethical considerations. This may have affected the results; however, none of the controls had chronic disease and their biopsy histology was normal.

Cow's milk protein tolerance tests

The children in Studies IV and V were considered to have delayed-type CMA on the basis of the open challenges. A blinded challenge would have been scientifically valuable but was felt to be too laborious for clinical work. On the other hand, a double-blind placebo-controlled setting was used in Study VI, and only one milk-hypersensitive subject was found among the young adults. It is possible, either that an open challenge may overestimate the presence of CMA or that delayed CMA may be present in school-aged children but not in young adults.

None of the children of Studies **IV** and **V** had the obvious and immediate symptoms often seen in infants and young children with IgE-mediated CMA. Earlier studies with school-aged patients have included comprehensive measurements with the skin prick test, an atopic patch test, milk-specific IgE and atopic changes in histology (eosinophilia), and in almost all cases the results have been negative and have not correlated with the results of the cow's milk challenge (Kokkonen et al. 1999, Kokkonen et al. 2001c, Kokkonen et al. 2001b). For this reason, in this study series total and milk-specific IgE has not been routinely measured in patients who have delayed reactions.

Lactose maldigestion tests

Digestion of lactose was measured by several methods. The symptomatic response to the ingestion of lactose was carefully monitored by means of written symptom records, and subjects with hypolactasia were diagnosed as being lactose intolerant according to their symptomatic response (**I**, **II**, **VI**). In Study **I**, the digestion of lactose was measured by a hydrogen breath test, which is reliable and non-invasive because no blood samples are required (Peuhkuri et al. 1998). However, the microbiota of some subjects may produce methane from hydrogen and so, in order to exclude erroneous negative results, in Study **II** the methane breath test was combined with the hydrogen breath test. The hydrogen breath test was considered too time-consuming for Study **VI**, which had a large study population, and therefore the alcohol-galactose test was used (Isokoski et al. 1972). Because of the young age of the subjects, as low an alcohol intake as possible was chosen, and the subjects ingested 150 mg of alcohol per body kg, the amount considered optimal by Pelto et al. (2000). Seven subjects, whose religious beliefs forbade the consumption of alcohol, took part in a conventional lactose tolerance test with serum glucose measurements. Because of the relatively low alcohol intake and the two different lactose challenge methods used, in Study **VI** both the symptomatic subjects and the controls were genotyped for the C/C_{-13910} variant of lactase persistence/nonpersistence (adult-type hypolactasia) (Enattah et al. 2002, Kuokkanen et al. 2003, Rasinperä et al. 2004). The subjects of Studies **IV** and **V** were patients of Oulu University Hospital, and

when lactose intolerance was suspected, their lactose absorption was analysed by a routine lactose tolerance test with serial serum glucose measurements, as is customary in Finnish hospitals.

Immunologic analysis

The local cytokine environment of the intestine was investigated by rt-PCR analysis of biopsy specimens (**V**, **VI**), and ELISA and cytometric bead array analysis of incubated biopsy supernatants (**IV**). Intestinal cytokines are usually studied by immunohistochemistry or *in situ* hybridisation, which reveal the number of cytokine-specific cells in the tissue. Rt-PCR analysis of cytokines in biopsy samples reflects the activation of cytokine-specific genes but the results do not always correlate with those from immunhistochemistry or *in situ* hybridisation. It is possible that the amount of cytokine-specific cells in tissue does not directly correlate with the amount of cytokine secreted since the method does not reflect the activity of the cytokine-secreting cells. The amount of cytokine, or the balance between the cytokines in the tissue, is thus difficult to estimate with these methods. In Study **IV**, the release of cytokines from biopsy samples was measured in order to obtain a picture of the network of cytokines secreted in the intestinal environment. Since the biopsies were not stimulated with any mitogen or antigen during incubation, the result emphasises the biological significance of these intermediates.

2 EFFECTS OF MILK HOMOGENISATION (I-III)

No differences in the symptoms during the challenges with homogenised and unhomogenised milk, studied in three different settings (**I**, **II**), were discerned. Nor, in Study **III**, did antibody production by milk-tolerant adults differ between the challenges with homogenised and unhomogenised milk. Our findings accord with the data of previous double-blind placebo-controlled trials, in which no difference between homogenised and unhomogenised milk has been shown in the symptoms of cow's milk allergic children (Hansen et al. 1987) or in the symptoms and complement-receptor expression of milk hypersensitive, lactose-intolerant or control subjects (Pelto et al. 2000). In animal studies, homogenised milk has been found to cause more intensive allergic reactions than unhomogenised milk (Poulsen et al. 1987, Nielsen et al. 1989, Feng & Collins 1999), and our results do not exclude the possibility that homogenised and unhomogenised milk could induce different types of primary immunisation to cow's milk antigens in immunologically intact infants.

According to the interviews in Study **II**, in the milk interventions of Studies **I** and **II** the 2-5-day milk challenges should have been long enough to produce symptoms, and the 5-10-day wash-

out periods between the challenges, long enough to prevent any carry-over effect. However, in Study **I**, most of the subjects experienced more symptoms during the second challenge than during the first, regardless of which milk they were consuming. The carry-over effect did not cause this, because during the wash-out period between the challenges the subjects did not experience any more symptoms than during the run-in period. None of the subjects in Study **I** were lactose intolerant, but they all experienced adverse gastrointestinal reactions during the milk challenges. It is possible, therefore, that some subjects who related their symptoms to milk homogenisation may have delayed-type cow's milk-protein allergy reacting in a dose-response manner, and it can be hypothesised that the second milk challenge exceeded their tolerance limit. On the other hand, in Study **II**, the subjects experienced more symptoms during the first study milk period than during the second, irrespective of which milk they were ingesting. It has been suggested that lactose-intolerant subjects become accustomed to small amounts of lactose in their diet (Pribila et al. 2000), which could partially explain this finding.

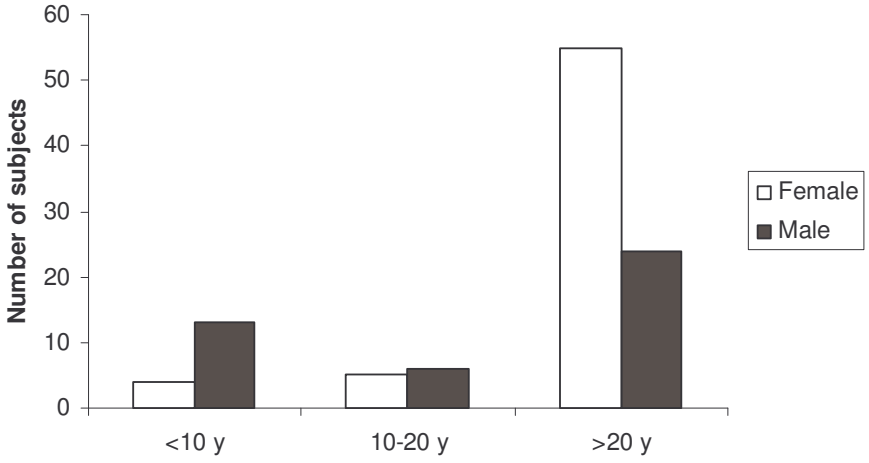

Figure 17 Number of females and males subjectively reporting intolerance to homogenised milk (II).

In the interviews in Study **II**, it was found that most subjects with subjective experience of a better tolerance of unprocessed than processed milk used small quantities of homogenised dairy products in their diet, either despite symptoms or even without symptoms. Most had found some dairy products they were able to consume without symptoms, and therefore immunological CMA cannot be the reason for their symptoms. This accords with a previous interview study, in which subjects with subjective intolerance to homogenised milk were able to consume some milk products without symptoms (Lagström et al. 2003). Interestingly, most of those children interviewed who were subjectively sensitive to processed milk were boys, whereas most of the adults inter-

viewed were women (Fig. 17). The male sex is known to be immunologically weaker during childhood (Sears et al. 1993); on the other hand, functional gastrointestinal diseases seem to be more common among women than among men (Naliboff et al. 2003), which could partially explain our finding.

In conclusion, these results do not show any significant difference in the tolerance of homogenised or unhomogenised cow's milk, either in subjects with self-reported symptoms suggestive of hypersensitivity to homogenised milk or in lactose-intolerant subjects, and no difference in cow's milk protein-specific antibody responses to homogenised and unhomogenised milk in milk-tolerant adults. The symptoms of the intolerant subjects were real but not affected by milk processing. Therefore there is no physiological reason to recommend unhomogenised milk to lactose-intolerant individuals, or to other symptomatic individuals.

3 IMMUNOLOGICAL FINDINGS IN DELAYED-TYPE COW'S MILK ALLERGY (IV, V)

Studies **IV** and **V** showed unique intestinal immune activation in delayed-type CMA, characterised by the up-regulation of both Th1- and Th2-type cytokines and a slight increase in the number of intraepithelial T cell populations.

Cytokine network in delayed-type CMA

The results of Studies **IV** and **V** concerning intestinal cytokine profiles in delayed CMA indicated that both Th1 and Th2 lymphocytes were locally activated in the intestine of these children, which suggests that delayed CMA is a separate entity and is different from atopic food allergy. This hypothesis accords with a study by Hauer and co-authors (1997), in which patients with cow's milk sensitive enteropathy (CMSE), a local delayed-type hypersensitivity, showed increased frequency of both IFN-γ- and IL-4-secreting lamina propria lymphocytes on duodenal mucosa compared to the controls, as detected by ELISPOT. In that study, both the children with CMSE and those with CMA showed increased IFN-γ, IL-4, IL-5 and IL-10 ELISPOTs in the blood compared to the controls. However, IL-4 ELISPOTs were greater in the children with CMA compared to those with CMSE, which indicates differences in immediate and delayed reactions towards milk.

Our finding of increased IFN-γ release in children with delayed CMA (**IV**) accords with a study by Veres et al. (2003), in which children with untreated delayed-type food allergy to cow's milk and/or cereals had elevated densities of IFN-γ cells and high expression of IFN-γ mRNA in

the duodenum, detected by immunohistochemistry and *in situ* hybridisation methods. These findings suggest that the activation of the Th1 immune response is associated with gastrointestinal hypersensitivity. However, in Study **V**, we could not demonstrate increased expression of IFN-γ mRNA in delayed CMA. It may be that the amount of cytokine-specific mRNA does not correlate with the actual production of the protein. This is supported by a study in which IFN-γ-secreting cells dominated in the Peyer's patches and the ileal lamina propria in healthy children who had no signs of gastrointestinal disease, but the mRNA levels of IL-4 and IL-10 were either higher or equivalent to IFN-γ mRNA (Hauer et al. 1998). This suggests that, at least in healthy intestinal mucosa, the number of IFN-γ-secreting cells is higher than that of IL-4- or IL-10-secreting cells, the last two, however, being strongly activated and expressing abundant mRNA.

The increased secretion of IL-4 in Study **IV**, and the increased expression of IL-6 and CCR-4 mRNA in Study **V** supports the view that in delayed CMA the Th2 lymhocytes are activated, too. CCR-4 is a chemokine receptor expressed in Th2 lymphocytes and associated with hypersensitive states (Sallusto et al. 1998, Imai et al. 1999), and IL-6 supports the so-called Th2-type immune response, since it activates IL-4-secreting cells and contributes to mucosal IgA production (Beagley et al. 1989, Rincon et al. 1997). Markers of Th2 lymphocyte activation suggest that these children may still show some signs of local IgE-mediated reactions, since many of them had an IgE-mediated allergy in infancy. Indeed, Lin et al. (2002) found reduced numbers of IFN-γ^+ cells and increased numbers of IL-4^+ cells, detected by immunostaining, in duodenal biopsies of symptomatic food-allergic adults with negative serum IgE, suggesting a localised IgE-mediated response in the gastrointestinal tract during the symptomatic period of these patients.

The activation of Th2 lymphocytes indicates a failure to suppress the immune responses against luminal cow's milk antigens to tolerance. However, in Study **V**, the mRNA expression of regulatory cytokine TGF-β showed no difference, either in delayed CMA or in celiac disease, compared to the controls. On the other hand, when the children in Study **IV** who had delayed CMA were divided into different diet groups, those who avoided cow's milk showed up-regulation of TGF-β. The decreased release of TGF-β among those who were exposed to dietary cow's milk supports earlier findings of the down-regulation of TGF-β in young children with food allergies (Pérez-Machado et al. 2003). TGF-β, produced by regulatory T cells, is an inhibitory cytokine recognised as a key regulator of immunological homeostasis and inflammatory responses, and is associated with the development of oral tolerance (Yazdanbakhsh et al. 2002, Umetsu et al. 2003). Interestingly, an increase of IL-10 was also seen in those children with delayed CMA who consumed milk (**IV**). IL-10 is a cytokine with a dual function: it may have anti-inflammatory activity and inhibit Th1 and Th2 responses, and it is also a mediator of T regulatory cell activities. The

anti-inflammatory role of IL-10 in the intestine has recently been questioned, and the protective effect of IL-10 may merely be an indirect consequence of its effect on TGF-β secretion (Fuss et al. 2002). In Study **IV**, the release of IL-10 correlated with the levels of IFN-γ in all the study groups, which suggests that IL-10 reflects inflammation in the human mucosa, as earlier reported in studies on rheumatoid arthritis and spondyloarthropathy (Van Damme et al. 2001, Nissinen et al. 2004). Thus, our finding of up-regulated IFN-γ and IL-10 may indicate intestinal inflammation and disorder in the permeability of the mucosa in children with delayed CMA.

Endoscopic findings and intraepithelial T cells in delayed CMA

The endoscopic, histological and imunohistochemical findings of Studies **IV** and **V** confirm previous findings concerning the mucosal state in the duodenum and ileum of patients with delayed CMA (Kokkonen et al. 2000, Kokkonen et al. 2001b). Lymphonodular hyperplasia of the intestine has been reported in children with gastrointestinal symptoms (Kokkonen & Karttunen 2002, Kokkonen et al. 2002), and this was also a typical finding in the children of our Studies **IV** and **V**. This seems not to be the case among young adults, as none of the symptomatic young adults of Study **VI** had LNH of the duodenal bulb or the terminal ileum.

A massive increment of intraepithelial $CD3^+$, $\alpha\beta^+$ and $\gamma\delta^+$ T cells is a typical characteristic of celiac disease (Kontakou et al. 1995, Westerholm-Ormio et al. 2002, Veres et al. 2003), as was seen in the children with celiac disease in Studies **IV** and **V**, and was also reported to a lesser extent in food-induced gastrointestinal hypersensitivity (Spencer et al. 1991, Savilahti 2000, Kokkonen et al. 2001b). In Study **IV**, the densities of the intraepithelial T cells were significantly elevated in those children with delayed CMA who were exposed to cow's milk but not in those who avoided it, which reflects the down-regulation of intestinal immune activation in the treated disease. In Study **V**, we found mild elevation in the densities of intraepithelial $\gamma\delta^+$ T cells in the duodenum, and highly elevated densities of $\gamma\delta^+$ T cells and the $\gamma\delta^+/CD3^+$ ratio in the ileum of delayed CMA patients. In Study **V**, the up-regulation of cytokine expression was also most pronounced in the samples from the terminal ileum, where the signs of intestinal hypersensitivity (i.e. prominent LNH and an increase in $\gamma\delta^+$ T cells) were most prominent in the children with delayed CMA. In delayed CMA, no increase of mononuclear cell infiltration in the lamina propria, no villous atrophy and no accumulation of immune cells outside the germinal centres was observed either in duodenal or in ileal biopsy samples (**IV**, **V**).

4 FINDINGS IN IMMUNE-LIKE GASTROINTESTINAL SYNDROME (VI)

The main aim of Study **VI** was to evaluate the occurrence of hypersensitivity to cow's milk protein in young adults with gastrointestinal complaints similar to those described in younger children in Studies **IV** and **V**. However, we were not able to find even one case with lymphonodular hyperplasia. In a blinded challenge, cow's milk yielded the same amount of symptoms as the placebo soy drink.

Almost half the subjects reported having suffered some abdominal complaints during the previous year, but in most cases the symptoms appeared infrequently and did not disturb their normal life. In Study **VI**, in agreement with earlier studies on children (Kokkonen et al. 2001b, Kokkonen et al. 2004), 10% of the young adults reported intensive and major gastrointestinal complaints. The frequency of gastrointestinal symptoms subjectively related to consumption of cow's milk was surprisingly high, a quarter of all the subjects. Lactose intolerance was present in 13% of the study subjects, and 3% claimed to have milk allergy, both the intolerance and the allergy being, in most cases, self-diagnosed. Pelto et al. (1999) calculated that the frequency of cow's milk protein hypersensitivity is 3-6% among young adults in Finland, while the prevalence of lactose intolerance was estimated to be about 6%. Our negative results from the blinded milk-protein challenge and the hypolactasia gene test do not support these estimates. In the placebo-controlled low-lactose milk challenge, milk protein was found to cause pronounced symptoms in only two (one of whom was lactose intolerant) of the 23 subjects who completed the challenge, and four subjects out of the 47 symptomatic subjects had the C/C_{-13910} genotype associated with low lactase activity (Enattah et al. 2002, Kuokkanen et al. 2003, Rasinperä et al. 2004). According to these observations there seems to be a very marked discrepancy between self-observed food-related reactions and definitely diagnosed hypersensitivity or intolerance states as demonstrated by many earlier investigators (Suarez et al. 1995, Vesa et al. 1998, Vernia et al. 2004). The problem is that the avoidance of milk and dairy products by symptomatic subjects, also seen as decreased levels of cow's milk specific antibodies, often leads to impaired nutrient intake (Weinberg et al. 2004).

Although cow's milk did not trigger gastrointestinal symptoms, our results suggest that inflammatory mechanisms are involved in food-related gastrointestinal complaints, because, compared to the controls, the symptomatic cases as a group showed higher concentrations of circulating sICAM-1 and a tendency towards the up-regulation of TGF-β and IL-12p35 mRNA expression in the mucosal biopsies. IL-12 is a monocyte-derived cytokine which enhances IFN-γ up-regulation. Soluble ICAM-1, induced by IFN-γ, is important for eosinophil and neutrophil adhesion, and a high concentration of sICAM-1 in the plasma has been observed as a marker of persis-

tent airway inflammation in asthmatic patients (Kokuludag et al. 2002). Exposure to cow's milk during infancy also induces circulating sICAM-1, reflecting an inflammatory response to orally-ingested foreign proteins (Paronen et al. 1996). To conclude, the subjects with food-related gastrointestinal symptoms had increased activation of the immune system and markers of inflammation of the intestinal mucosa. Supporting this, the symptomatic patients tended to have higher counts of intraepithelial $CD3^+$ T cells in the duodenal mucosa compared to the controls.

In conclusion, 10% of the young Finnish adults reported major gastrointestinal complaints, 24% reported cow's milk-induced gastrointestinal symptoms, and 13% did not drink milk at all in its primary, liquid form. However, in a blind challenge, cow's milk protein-induced symptoms were rare and similar to those of a placebo soy drink. Yet markers were found of skewed immune response on the gastrointestinal mucosa in the symptomatic subjects. The food-related gastrointestinal symptoms of young adults seem to be caused by the unspecific and unknown traits of an altered mucosal immune response rather than being triggered by cow's milk, as is often suspected by the patient. This new entity of intestinal immune-mediated disorder may be a self-perpetuating disease showing fluctuations of symptoms. An autoimmune nature of the state, at least in a subgroup of the affected subjects, cannot be ruled out, and this hypothesis is supported by the observation that the HLA DQ*02 allele predisposing to autoimmunity was almost twice as common among the symptomatic individuals as among the rest.

CONCLUSIONS

In the present study, the effects of cow's milk and its processing on gastrointestinal symptoms and delayed-type, (i.e. non-IgE-mediated), immune responses were studied. Based on the results presented in this thesis, the main conclusions are as follows:

1. Homogenised and unhomogenised cow's milk was tolerated equally by subjects with self-reported symptoms suggestive of hypersensitivity to homogenised milk, and by lactose-intolerant subjects. Nor was any difference in the production of cow's milk protein-specific antibodies discerned in milk-tolerant adults during the challenges with homogenised and unhomogenised cow's milk. The hypersensitivity reactions of the intolerant subjects were real but the processing of milk did not affect the symptoms. The possibility cannot be ignored that some individuals may benefit from unprocessed milk, but there is no evidence-based physiological reason to recommend unhomogenised milk to lactose-intolerant subjects in general, or to other symptomatic individuals.

2. Children with non-IgE-mediated, delayed-type CMA showed characteristic local cytokine activation in the intestine, consisting of the high release of IFN-γ and the up-regulated expression of IL-6 and CCR-4 mRNA combined with some evidence of a reduced IL-2 mRNA expression. These results suggest that delayed-type CMA is a local intestinal immune-activation state showing activation of both Th1 and Th2 lymphocytes.

3. No evidence of an occurrence of similar hypersensitivity to cow's milk protein as described in younger children was found in young adults with gastrointestinal complaints. In a blind challenge, cow's milk protein-induced symptoms were rare and similar to those of a placebo soy drink. However, markers of skewed immune response on the gastrointestinal mucosa in the symptomatic subjects were found. The food-related gastrointestinal symptoms of the young adults seemed to be caused by the unspecific and unknown traits of an altered mucosal immune response rather than being triggered by cow's milk, the latter being often suspected by the patient. This new entity of intestinal immune-mediated disorder may be a self-perpetuating disease showing fluctuation of symptoms. An autoimmune nature of the state, at least in a subgroup of the subjects affected, cannot be ruled out, and this hypothesis is supported by the observation that the HLA DQ*02 allele predisposing to autoimmunity was almost twice as common among the symptomatic individuals as among the rest.

4. In a questionnaire, 10% of the young Finnish adults reported major gastrointestinal complaints, 24% reported cow's milk-induced gastrointestinal symptoms and 13% did not drink any milk as such. However, in a blind challenge only one young adult was found to be hypersensitive to cow's milk. According to these observations there seems to be a very marked discrepancy between self-observed food-related reactions and definitely-diagnosed hypersensitivity or intolerance states.

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ORIGINAL PUBLICATIONS

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